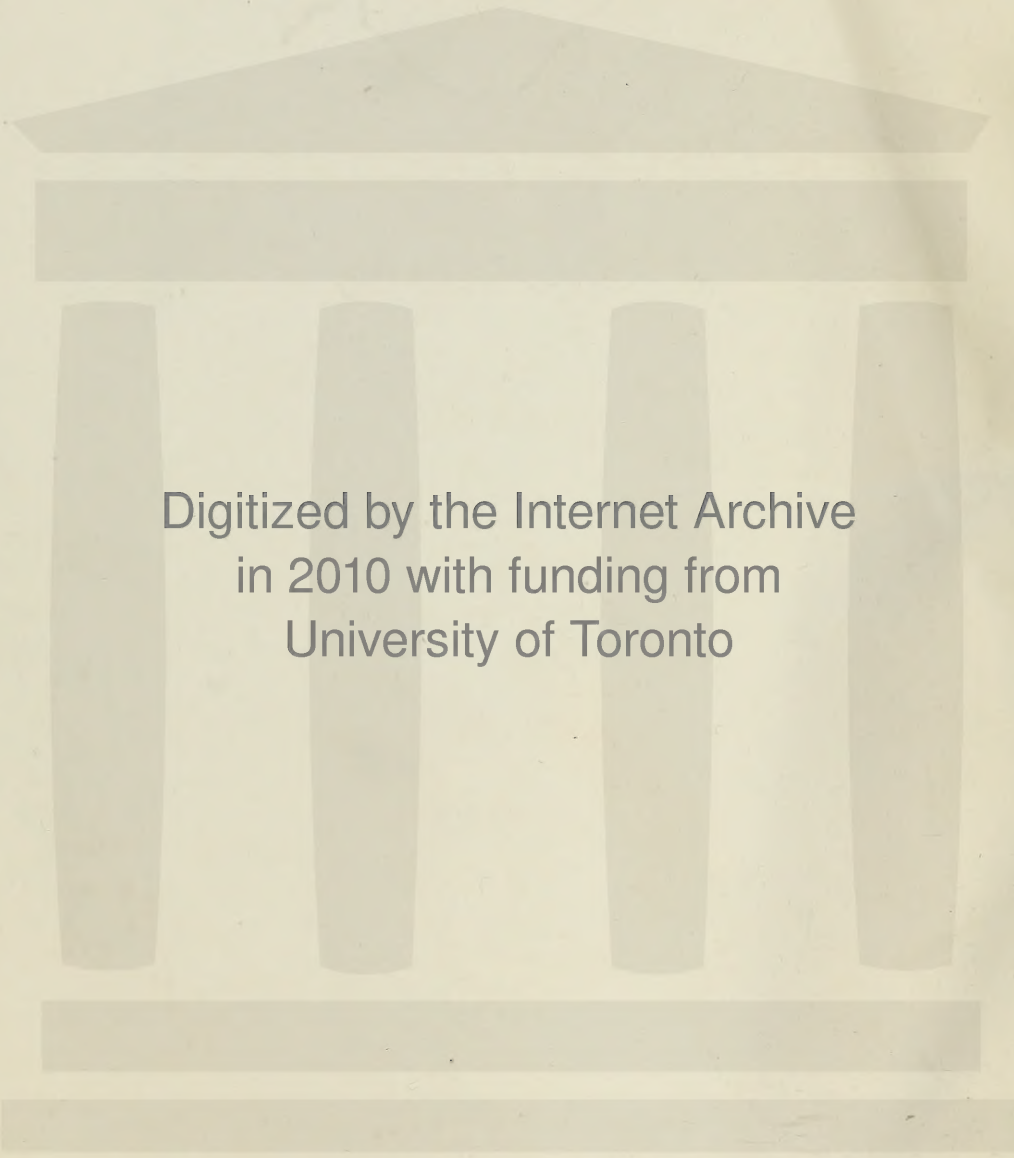


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AUTOHEMAGGLUTINATION EXPERIMENTALLY INDUCED BY THE REPEATED WITH- DRAWAL OF BLOOD.

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PLATE 22.

(Received for publication, March 11, 1918.)

There is much evidence in the literature to show that moderate losses of blood act to increase the titer of antibodies developed in response to an injected antigen.¹ Little attention, though, has been given to whether the production of antibodies normal to the organism is likewise stimulated. The point has considerable clinical interest, especially in connection with the remarkable resistance to infection manifested by many patients with severe anemia. In the work now reported one phase of the problem has been taken up; namely, the influence of repeated bleedings on the normal isohemagglutinins.

Experiment 1.—Interagglutination tests were carried out with the cells and sera of twelve normal rabbits. The cells had been twice washed and made to 5 per cent suspensions with salt solution. The sera were obtained from the clot at room temperature (22°C). Mixtures of each serum with each cell suspension in equal parts were kept at 22°C. for 15 minutes and examined with the microscope. Agglutination was found in a moderate proportion of the 144 mixtures. Autoagglutination was not noted.

On the basis of the findings, the animals were separated into two groups, each possessing about the same proportion of material for isoagglutination; that is to say, susceptible cells and agglutinating sera. One group of six rabbits was set aside as controls, and the remaining six rabbits were bled 10 cc. from the heart every 3 to 6 days during a period of 2 months. All were kept under the same conditions. From time to time the interagglutination tests were repeated, sometimes by the method just described, sometimes by mixing the whole citrated bloods in the proportions of 9 to 1 and 1 to 9, according to the method of Rous and Turner.²

¹ See Lippmann, *Z. exp. Path. u. Therap.*, 1914, xvi, 124, for large bibliography.

² Rous, P., and Turner, J. R., *J. Am. Med. Assn.*, 1915, lxiv, 1980.

Experiment 2.—Fourteen rabbits were used in two groups arranged on the same basis as in Experiment 1, but with only six individuals in the control group. The other eight animals were bled as already described during a period of 26 days. Interagglutination tests were carried out between the control animals and between these and the bled animals, but not between the individuals of this latter sort. Citrated bloods were used in the tests, which were repeated at intervals of a week or more. Tests with sera and washed cells were also made when this seemed advisable. In whole citrated bloods autoagglutination is easily seen. None was discoverable prior to the bleedings.

No Induced Isoagglutinins.

A number of the rabbits possessed isoagglutinins to start with. Some were bled, and some kept as controls. The bleedings had no demonstrable effect to alter the isoantibodies or to cause an appearance of new ones. It is true that weak isoagglutinins sometimes developed in individuals possessing none to begin with, but they were found to practically an equal degree in the controls and were probably in the nature of intercurrent serum changes such as Ottenberg and Thalhimer³ have reported.

Clumping in the Shed Blood.

In five out of fourteen of the bled rabbits there developed a tendency of the red cells to clump together into masses in the shed blood. The clumping was never general, bringing together all the cells, as in the case of rabbits repeatedly transfused,⁴ but the cell masses lay scattered here and there amid free cells. The phenomenon was not found in any of the twelve controls, nor has it since been observed in a large series of other normals. In instances of anemia, on the other hand, resulting from malnutrition, it has sometimes been met with.

The clumping phenomenon was definitely associated with the anemia following in some instances on the bleedings. Many medium sized rabbits withstand excellently the loss of 10 cc. of blood every 3 to 5 days during a long period. Their hemoglobin percentage and the appearance of the corpuscles remain practically unaffected. The clumping was never noted in these animals. In other rabbits the

³ Ottenberg, R., and Thalhimer, W., *J. Med. Research*, 1915-16, xxxiii, 213.

⁴ Rous, P., and Robertson, O. H., *J. Exp. Med.*, 1918, xxvii, 509.

bleedings rapidly brought about a moderate anemia with pale corpuscles, microcytes, and poikilocytes in circulation. In such instances the clumping occurred, though it was by no means a regular accompaniment of the condition. The number of bleedings and total loss of blood prior to appearance of the clumping were sometimes surprisingly small. Clumping was well marked in a 1,500 gm. rabbit 3 days after the last of two bleedings of 10 cc. each with an interval of 3 days between. In another animal of 1,350 gm., also bled twice, but at an interval of 7 days, the phenomenon was visible 3 days after the last bleeding. In both cases the blood loss was very poorly borne.

The clumping was plainly apparent in whole citrated bloods⁵ allowed to stand for 15 minutes or more at room temperature and examined microscopically after dilution with salt solution. When at all marked it could be seen in thick slide preparations of the blood, as such, providing the cells were not numerous enough to interfere with the observations (Fig. 1). Under these circumstances it appeared within about a minute after the blood was shed and before any clotting had occurred. Each clump consisted of from 3 or 4 to 40 or 50 corpuscles massed helter-skelter.

Cause of the Clumping.

The question whether the phenomenon had its cause in a change in the cells, or plasma, or in both, was largely answered by the routine tests of Experiments 1 and 2. These demonstrated that the cells of the bled rabbits had undergone no alteration as regards agglutinability or inagglutinability by normal sera of known behavior. Furthermore, the clumping, like that due to the autoagglutinin present in normal plasma⁶ and the principle present in the plasma of transfused rabbits,⁴ did not occur at body heat. The cells remained free in citrated blood at 38°C., and the massing together which was visible at room temperature disappeared when the specimen was warmed. Corpuscles separated from the citrated plasma while warm, then washed in warm salt solution and brought together in it at room temperature,

⁵ 10 parts of blood to 1 part of a watery 10 per cent solution of sodium citrate.

⁶ Landsteiner, K., *Münch. med. Woch.*, 1903, i, 1812.

failed entirely to clump. But when a little of the thick cell suspension was dropped into the original citrated plasma, the cells massed together at once. All these facts showed that the clumping was due to an element in the serum and that this element has much in common with the normal and induced autoagglutinins.

Distinguishing Traits of the Agglutinin.

The agglutinin of the bled rabbits was able to cause clumping in the whole blood as such, or in the whole citrated blood, at room temperature ($22^{\circ}\text{C}.$), whereas the normal agglutinin is effective at 22° only when a large amount of serum is allowed to act on a few cells.⁵ The agglutinating principle of the bled rabbits, obtained in the free state, as in serum separated from the cells by defibrination and centrifugation at $38^{\circ}\text{C}.$, was so strong in all cases as to bring about clumping at $22^{\circ}\text{C}.$ in mixtures of the serum with an equal part of a 5 per cent suspension of the animal's own washed cells, and in some instances when there was a further dilution with one part of salt solution. The serum of five normal rabbits similarly separated and tested yielded not the least trace of agglutination. These results with a constant amount of antigen (the 5 per cent cell suspension) rule out the possibility that clumping in the anemic rabbits was due merely to the action of the normal autoagglutinin on an antigen (the red corpuscles) diminished in quantity by the bleedings.

Attempts to obtain the agglutinating factor in salt solution led to a singular finding. Normal autoagglutinins become much more effective as cooling proceeds from room temperature to $0^{\circ}\text{C}.$ and are best demonstrated in the cold. The agglutinin of the bled rabbits, on the other hand, while effective at room temperature, may be relatively little enhanced in activity by further cooling, and at $0^{\circ}\text{C}.$ may be surpassed in its action by the normal antibody.

Experiment 3.—A few cubic centimeters of blood were obtained from each of two normal rabbits and two which had been repeatedly bled and were the possessors of an agglutinin which caused clumping at room temperature. The sera were separated from the cells by defibrination in the warm, and centrifugation in a water jacket at $38^{\circ}\text{C}.$ Those of the bled animals caused clumping at room temperature when mixed with an equal volume of a 5 per cent suspension of the corresponding cells twice washed in the warm. In similar mixtures of normal

sera no clumping occurred. Now 1.2 cc. of each serum was mixed with 0.1 cc. of a 50 per cent suspension of the corresponding cells, and the tubes were plunged in melting ice. The results are given in Table I.

It will be observed that the agglutinin of the normal rabbit No. 2, while practically ineffective at room temperature (22°C.), caused a more complete clumping at 0°C. than did the agglutinins of the bled rabbits, which were so active at 22°C. A second experiment along these lines gave similar results. The data do not enable one to say whether two distinct sets of antibodies are here concerned, but they

TABLE I.

Rabbit.	Room temperature (22°C.). Microscopic observations after 15 min.				0°C. Macroscopic observations.	
	Whole blood.	3 parts serum+ 1 part 5 per cent red cells.	1 part serum+ 1 part 5 per cent red cells.	1 part serum+ 1 part salt so- lution+ 1 part 5 per cent red cells.	1.2 cc. serum + 0.1 cc. 50 per cent red cells.	
					After 8 min.	After 66 min.
Normal.						
1	0	0	0		+	Sedimentation incomplete; sedi- ment finely granular.
2	0	Tr.	0		++++	Complete sedimentation into a sin- gle, solid mass.
Bled.						
3	+ -	+	Tr.		+++	Sedimented completely into fairly large masses.
4	+		+	Tr.	++++	Complete sedimentation into a few large masses.

show clearly that the effects of an autohemagglutinin at one temperature cannot safely be taken as the indicator of the effects at another. The study of such antibodies assumes in consequence great complexity.

Agglutination and Rouleau Formation Are Not Connected.

The observations of several authors have led them to conclude that rouleau formation is intimately connected in its cause with agglutination. Our findings in transfused rabbits⁴ would seem also to point to this, since the appearance of new agglutinins in the blood is

always preceded by exaggerated rouleau formation. But the present results with rabbits repeatedly bled prove that the association is not obligatory. Here a partial or complete loss of the tendency to rouleau formation was regularly noted to accompany the development of the autoagglutinin (Fig. 1).

SUMMARY.

The repeated withdrawal of moderate quantities of blood does not lead to a development of new isoagglutinins in rabbits, or to noteworthy changes in normal ones already present. On the other hand, clumping of the animals' own cells in specimens of the whole blood is a frequent result. It is found in animals rendered anemic by the bleedings, not in those that rapidly repair their losses and remain in good condition. A similar clumping is sometimes to be seen in the blood of rabbits rendered anemic by malnutrition.

The clumping is due to a true autoagglutinin, which differs from the normal autoagglutinin in its far greater strength, as also, at least in some instances, by a peculiar variation in its activity with changes of temperature.

In the rabbits which developed isoagglutinins after bleeding, the tendency of the cells to form rouleaux was far less than the normal. It follows that rouleau formation is not essentially connected with autoagglutination, as has been assumed in the past.

In the light of the present findings a systematic search for autohemagglutinins in the blood of anemic patients would seem of interest. They have been noted in sick human beings (Ascoli, Klein), but not in recent years. The reason for this may well be that present day blood examinations are not of a sort to bring about their discovery. Thick films of fresh blood are seldom used for clinical purposes, and it is in thick preparations that clumping is most readily observed.

EXPLANATION OF PLATE 22.

Fig. 1. Autoagglutination in the blood of a rabbit rendered anemic by bleeding. Fresh slide-and-cover-glass preparation.

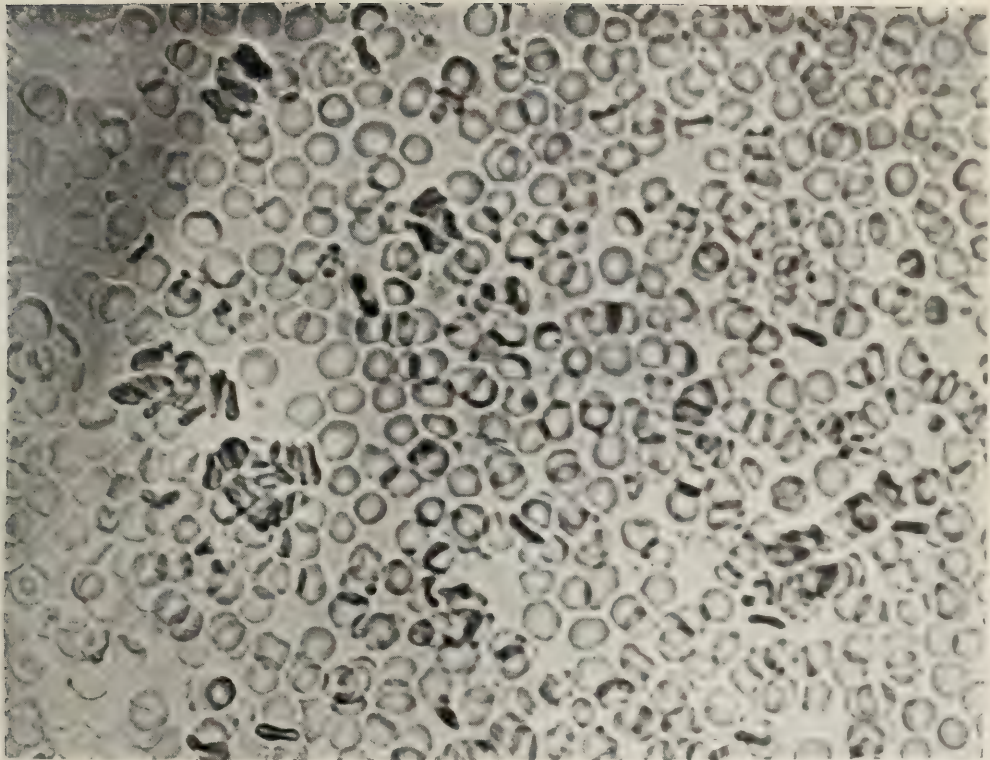
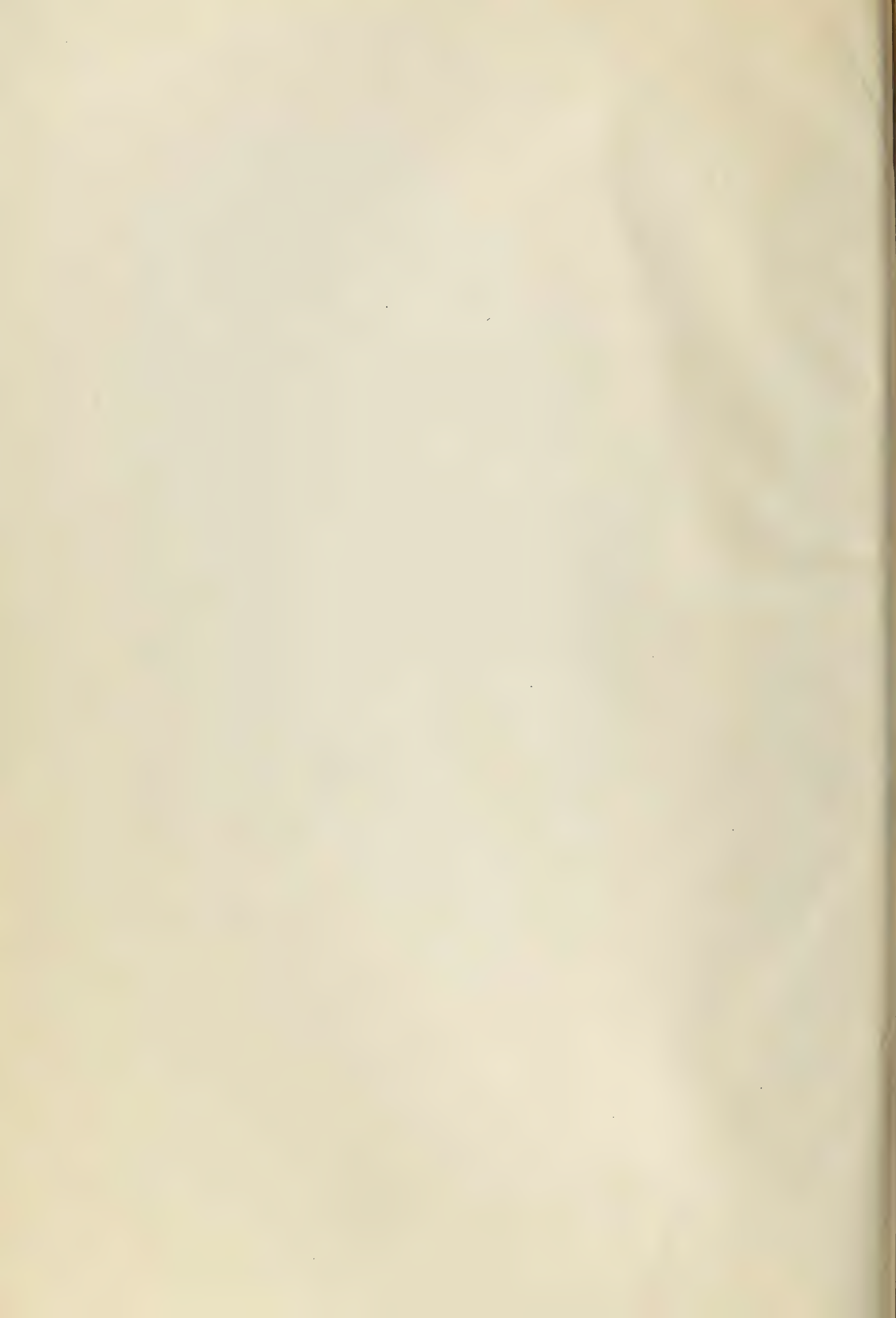


Fig. 1.

(Robertson and Rous: Autohemagglutination.)



MORPHOLOGICAL CHARACTERISTICS AND NOMENCLATURE OF LEPTOSPIRA (SPIROCHÆTA) ICTERO-HÆMORRHAGIÆ (INADA AND IDO).

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 25 TO 29.

(Received for publication, February 27, 1918.)

In a previous communication, the writer reported the presence in American wild rats of a spirochete morphologically and immunologically identical with the *Spirochæta icterohæmorrhagiæ* of Inada and his associates and also with the strain isolated by Stokes from cases of infectious jaundice among British soldiers in Flanders.¹ The European strains, which have now been isolated from cases on the British, French, and Italian fronts, as well as from wild rodents captured not only near the battle-lines but in regions remote from them, are undoubtedly strains of the same organism.² Jobling and Eggstein³ have also found the same spirochete recently among wild rats caught in Tennessee.

Just how, in nature, a rat becomes a carrier of the spirochete is not at once apparent. It is not improbable that the contamination of a foodstuff by the urine of an infected rat may transmit the organism to other rats; or the animal may become infected by feeding upon an infected dead rat, since a rat may be experimentally infected by feeding it with an infected foodstuff or with an infected tissue or

¹ Noguchi, H., *J. Exp. Med.*, 1917, xxv, 755.

² Costa, S., and Troisier, J., *Presse méd.*, 1916, lxxx, 526, 565. Courmont, J., and Durand, P., *Bull. et mém. Soc. méd. hôp.*, 1917, xli, series 3, 115. Clément, P., and Fiessinger, N., *Presse méd.*, 1916, lxxx, 598. Garnier, M., *Compt. rend. Soc. biol.*, 1916, lxxix, 928. Manine, Cristau, and Plazy, *Compt. rend. Soc. biol.*, 1917, lxxx, 531. Wilmaers, L., and Renaux, E., *Arch. méd. Belges*, 1917, lxx, 115, 207. Dawson, B., and Hume, W. E., *Quart. J. Med.*, 1916-17, x, 90. Zironi, A., and Capone, G., *Sperimentale*, 1917, lxxi, 298. Ascoli, M., and Perrier, S., *Gazz. osp.*, 1916, xxxvii, 1618. Sisto, P., *Sperimentale*, 1917, lxxi, 361. Siccardi, P. D., and Bompiani, G., *Ann. ig.*, 1917, xxvii, 609. Moreschi, C., *Policlinico, Sez. Prat.*, 1917, xxiv, 265. Sampietro, G., *Ann. ig.*, 1917, xxvii, 23.

³ Jobling, J. W., and Eggstein, A. A., *J. Am. Med. Assn.*, 1917, lxix, 1787.

organ. Whatever the mode of preservation in nature, *Spirochæta icterohæmorrhagiæ* is a common commensal among rodents.

Morphology.

The morphology of this organism has been the subject of much study by its discoverers and by others, but its distinctive feature does not seem to have been recognized. Inada and his associates described the organism as a spirochete with several irregular waves, the entire body being dotted with alternate bright and shadowy portions.⁴ Hübener and Reiter, who described a similar picture, apparently believed that the organism had a series of minute knots, and hence gave it the name *Spirochæta nodosa*.⁵

That these investigators overlooked the true structure must have been due either to the difficulty of observing the organism, even under a powerful dark-field illumination, or to the indistinctness of the minute spirals in a stained preparation. It appears as an almost smooth bodied, wavy organism, not unlike *Spironema refringens* when fixed in methyl alcohol and stained with Giemsa's solution (Figs. 1, 2, and 3). As has been said in a previous paper,¹ the natural features of the organism can be well preserved when it is fixed in osmic vapor and then stained over night with Giemsa's solution. In such a preparation it is stained light purple and is seen to consist of a very tightly and regularly wound cylindrical filament tapering to sharply pointed extremities. The filament usually assumes a graceful hook at one or both ends, while the main portion may be straight or slightly bent (Figs. 4 and 5). The number of spirals (not waves) varies considerably according to the length of the specimen, which may be between 3 to 20, 30, or even 40 μ , but the distance between the apices of two spirals measures about 0.5 μ . For example, a specimen measuring 9 μ would have eighteen spirals. The thickness, or diameter of a cross-section, of the organism is nearly uniform until it approaches the terminal portion, which may be so conveniently designated because of its tapering points and its hooked attitude. The number of spirals in the terminal portion appears to

⁴ Inada, R., Ido, Y., Hoki, R., Kaneko, R., and Ito, H., *J. Exp. Med.*, 1916, xxiii, 377.

⁵ Hübener and Reiter, *Deutsch. med. Woch.*, 1916, xlii, 1.

be about six in all specimens, and it is this portion which exhibits the greatest tendency to become bent to a semicircle. Unlike various spironemata or treponemata, the spiral amplitude near the extremities is not noticeably less than that of the middle portion of the organism.

In certain specimens the terminal portions are far less intensely stained than the main portion (Figs. 1, 2, and 3). In the majority of specimens, both terminal portions are bent to the same side (Figs. 5, 6, 13, 16, 17, 19, and 22), but in some they form hooks of opposite direction (Fig. 4), unipolar hooks (Figs. 8, 9, 15, and 20), or are not bent at all (Figs. 11 and 12); and some are contorted (Fig. 18). In the less well preserved specimens the spirals are no longer distinct but appear as somewhat more deeply stained dots (Fig. 21). As has already been pointed out, under a powerful dark-field illumination the organism in rapid rotary motion seems to be surrounded by a halo. This may be only an optical effect, but a similar clear zone has been noticed in the stained preparations of some specimens (Figs. 12, 15, 17, and 23).

The dark-field picture of the organism is such that one may mistake the minute spirals for refractive beads arranged diagonally or somewhat obliquely with respect to the axis of the organism (Figs. 24 and 27 to 33), as originally depicted by Inada and his associates and others. But, as has been stated before, with a favorable and powerful illumination, the real structure can be revealed (Figs. 24, 25, and 34).

Only a few of the photomicrographs represent the characteristically hooked forms (Figs. 26 and 28) as actually seen in active rapid rotary motion in a free space, because it was difficult to photograph the organisms in motion, and as soon as motion ceases many of them lose the typical hooks. The large wavy undulations, however, (not the elementary spirals), as assumed by the organisms when penetrating semifluid medium, are well shown in some of the specimens at rest (Figs. 27 and 29 to 33). The remarkable flexibility of the organism in a semisolid medium is also shown (Figs. 27, 32, and 33). These minute filamental organisms dart through the soft medium with great rapidity, first in one direction and then in another, searching for a loose spot which they can pierce through. When encountering

an impenetrable obstacle they reverse their progression and start anew. A striking sight is thus presented by these little vermicular organisms darting in all directions. A vibratory motion of the free portion of the organism results when it is extricating itself from an entanglement. In an emulsion of infected liver one may encounter a tangle of several actively motile organisms (Figs. 24 and 25), while in a culture several weeks old a mass of hundreds of motile spirochetes may be found (Fig. 35).

The European (Figs. 36 to 57) and Japanese (Figs. 58 to 68) strains have all the morphological features given for the American strain. It might be mentioned here that the elementary spirals in the terminal portion are much smaller in number and less regular in the stained specimens of the European strain, but this may be due to imperfect fixation of the organism, because under the dark-field microscope the spirals are equally close and regular.

Classification.

Characteristics of Different Genera of Spiral Organisms.

In order to determine the systematic position of the organism of infectious jaundice, it may be well to review here the characteristics of various genera of spiral organisms. Through the recent investigations of Gross,⁶ Zuelzer,⁷ Dobell,⁸ Gonder,⁹ Swellengrebel,¹⁰ and others, the organism for which Ehrenberg created the term *Spirochæta* in 1838 is now known to be distinct from the majority of so called spirochetes. It consists of a long, highly flexible, central axial filament surrounded by a regularly wound layer of protoplasm, usually of great length (200 to 500 μ), and is free living in fresh or marine water (Fig. 108). Neither a membrane nor a flagellum is present. Multiplication takes place by transverse fission. The organism

⁶ Gross, J., *Centr. Bakteriolog., 1te Abt., Orig.*, 1912, lxxv, 83.

⁷ Zuelzer, M., *Arch. Protistenk.*, 1912, xxiv, 1.

⁸ Dobell, C., *Proc. Roy. Soc. London, Series B*, 1912, lxxxv, 186.

⁹ Gonder, R., *Spironemacea (Spirochaeten)*, in von Prowazek, S., *Handbuch der pathogenen Protozoen*, Leipsic, Liefg. 6, 1914, 671.

¹⁰ Swellengrebel, N. H., *Ann. Inst. Pasteur*, 1907, xxi, 448; *Compt. rend. Soc. biol.*, 1907, lxii, 213.

creeps along the surface of an object but does not swim. Only four species belonging to this genus have been described. The organism under discussion does not belong to it.

Cristispira and *Saprospira*.—For a limited variety of coarse, actively motile spiral organisms infesting the crystalline styles of certain mollusca, the genus *Cristispira* was proposed by Gross in 1910.¹¹ The characteristic features are: the presence of a membranous structure running spirally from one end of the body to the other, assuming the aspect of a crista or ridge; the chambered structure of the body; the absence of a terminal filament; and the existence of a strong, flexible membrane (Figs. 104 to 106). According to Gross, reproduction may be effected by multiple transverse fission or sporulation, though I have failed to confirm the occurrence of sporulation. More than a dozen species have been described, but from personal observations I doubt whether these so called species are sufficiently characteristic to be so distinguished. The type organism was first described by Certes in 1882¹² as found in oysters, and was known as *Spirochæta* or *Trypanosoma balbianii*. Another genus, *Saprospira*, was proposed by Gross in 1912¹³ for a few varieties of spiral organisms in mussels which differed from the cristispiræ in not having a crista (Fig. 107). The organism in question, however, belongs to neither of these genera.

Spiroplasma and *Treponema*.—Next in order is the large group of small parasitic spiral organisms commonly called spirochetes. Among them are the causative agents of syphilis and yaws (Figs. 69 to 72 and 103) and of relapsing fevers in man and animals (Figs. 94 to 100), non-pathogenic parasites in certain rodents, and various saprophytic types on or about the oral, alimentary, or genital mucous membranes (Figs. 73 to 93). Their essential feature is a spiral flexible body with terminal filaments, but no undulating membrane. They seem to multiply by transverse as well as longitudinal fission. The rigidity of the curves differs greatly in different organisms, some becoming almost flat at death or constantly changing the waves by oscillatory undulation, others retaining their regular curves even during motion or after death. The whole group has been called *Spirochætæ* or *Spirilla*, in spite of the

¹¹ Gross, J., *Mitt. zool. Station Neapel*, 1910-13, xx, 41.

¹² Certes, A., *Bull. Soc. zool. franc.*, 1882, vii, 347; 1891, xvi, 95.

¹³ Gross, J., *Mitt. zool. Station Neapel*, 1910-13, xx, 188.

fact that they have no affinity with the real spirochete or non-flexible spirillum. Gross includes them in the genus *Spironema*, a term introduced by Vuillemin¹⁴ in 1905 to distinguish Schaudinn's organism of syphilis from those with less rigid spirals. Dobell,⁸ however, believes that the term *Treponema*, as proposed by Schaudinn¹⁵ himself in 1905 for his organism, should be employed to designate all these minute parasitic varieties. Gonder⁹ takes a more conservative stand and upholds the distinction made by Schaudinn between the treponema type and that with less constant curves. For example, Gonder retains the genus *Spironema* for the latter and *Treponema* for the former type. I agree with Gonder in this respect, as the general features are sufficiently distinct to enable one to differentiate the two groups.

Nomenclature of Leptospira (Spirochæta) icterohæmorrhagiæ.

The striking differences between the organism of infectious jaundice and all the other so called spirochetes, or rather spironemata and treponemata, are apparent at a glance. The closely set, regular spirals of the organism of Inada and Ido remain unmodified during its rotary, spinning motions in a free space and when it is piercing a semisolid medium. While in motion in a free space, the whole body appears tightly drawn into a straight line, except for the usual hook formation of one or both terminal portions. When one end is extended and straight and the other semicircularly hooked, the organism usually progresses in the direction of the straight portion and seems to be propelled from the rear by the rotating hook (Figs. 8, 9, 15, and 20). A specimen with both ends hooked remains stationary in spite of its rapid rotary motions (Figs. 13, 16, and 19). By straightening one end or the other alternately, the organism changes its progression from one direction to the opposite one. When the organism penetrates a soft medium, changing direction very rapidly, it seldom shows hooked ends (Figs. 11, 12, 29, 30, 32, and 33). In this sort of movement the body assumes wide wavy undulations such as are seen in an active specimen of *Spironema refringens*. The behavior of the organism in semisolid medium is different from that

¹⁴ Vuillemin, P., *Compt. rend. Acad.*, 1905, cxi, 1567.

¹⁵ Schaudinn, F., *Deutsch. med. Woch.*, 1905, xliii, 1728.

in a free space. The persistence of the minute elementary spirals at all times is a feature which distinguishes this organism from any treponema or spironema. The depth of the spirals does not exceed the diameter of the body, a fact unknown among other so called spirochetes. A diligent search by means of various staining methods, as well as by dark-field illumination applied to cultures of different ages, has not demonstrated a terminal flagellum or peritrichal flagella or membranes. It is well to recall at this point that in old cultures of all the spironemata or treponemata I have isolated I have been able to demonstrate the presence of a terminal filament, even when it was observed with difficulty in uncultivated specimens. This organism, however, as far as we know at present, moves by means of its terminal portions. Moreover, unlike spironemata and treponemata, it withstands the action of 10 per cent saponin. Clearly it does not belong to either of these genera, but must remain in a class by itself until other similar organisms come to our observation. The nearest approach to it in morphological and biological respects is *Spirochæta biflexa*, which was isolated by Wolbach and Binger¹⁶ in 1914 from a filtrate of stagnant water taken from the shore of a fresh water pond near Boston. There is a great similarity between the two organisms. Both are filterable through Berkefeld filters. Wolbach and Binger did not succeed in obtaining a second generation in culture, and no tests of pathogenicity for experimental animals were made.

For the reasons which have been discussed, it seems justifiable to include the type of organism in question under *Leptospira* (λεπτός fine, + σπῆρα, coil), as has already been proposed.¹

The genera with their type organisms are presented below. The measurements of each of these representative members and the characteristic features used for identification of the genera are considered. There is little difficulty in distinguishing *Spirochæta*, *Saprospira*, *Cristispira*, and *Leptospira* from one another. But the distinction between *Spironema* and *Treponema* depends chiefly upon the rigidity and regularity of the spirals which are characteristic of the treponemata. Under natural conditions this difference is so marked that there should be no confusion in classification, but under cultural

¹⁶ Wolbach, S. B., and Binger, C. A. L., *J. Med. Research*, 1914, xxx, 23.

conditions the spirals of the spironemata acquire such rigidity and regularity that they, too, may be called treponemata. Dobell⁸ and Gross,⁶ independently of each other, and without any knowledge as to the morphological modifications due to cultivation, regarded the distinction between *Treponema* and *Spironema* as insufficient to maintain two separate genera, and Dobell chose the term *Treponema* and Gross *Spironema* for the same group of organisms. In my opinion the characteristics of *Treponema* and *Spironema*, under natural conditions, are sufficiently pronounced to justify retaining the two terms in classification. Neither *Treponema* nor *Spironema* has any feature which is likely to be confused with those of the other four genera referred to above. Text-fig. 1 shows the types mentioned below.

Genus.—*Spirochæta* (Ehrenberg, 1838). *Type Organism.*—*Spirochæta plicatilis* (Ehrenberg, 1838) (Fig. 108). *Measurements.*—Length, 100 to 500 μ ; blunt end. Diameter, 0.5 to 0.75 μ ; cylindrical. Spiral amplitude, 2 μ ; regular. Spiral depth, 1.5 μ ; regular. Waves, several, large, inconstant, irregular. *Axial Filament.*—Distinct in stained specimens; flexible; elastic. *Chambered Structure.*—Absent. *Membrane.*—Absent. *Crista.*—Absent. *Terminal Finely Spiral Filament.*—Absent. *Flagella.*—Absent. *Highly Motile End Portion.*—Absent. *Division.*—Transverse. *Habitat of Genus.*—Free living in fresh or marine water. *Other Species.*—*Plicatilis marina*, *plicatilis eustrepta*, *stenostrepta*, *daxensis*. *Staining Properties of Axial Filament and Cell Membrane.*—Axial filament consists of chitin or cutin-like substance. Stains violet by Giemsa's solution and gray by iron-hematoxylin. *Staining Properties of Body.*—Plasmic spirals of the body stain with eosin, rubin, etc. Contain volutin granules. *Trypsin Digestion.*—Axial filament resistant. *Bile Salts (10 Per Cent).*—Becomes shadowy pale but is not dissolved. *Saponin (10 Per Cent).*—Lives 30 minutes. Later becomes shadowy, but is not dissolved.

Genus.—*Saprospira* (Gross, 1911). *Type Organism.*—*Saprospira grandis* (Gross, 1911). *Measurements.*—Length, 100 to 120 μ ; obtuse end. Diameter, ? μ ; cylindrical. Waves, large, inconstant, shallow, irregular, 3 to 5 in number. Sometimes almost straight. *Axial Filament.*—Absent. *Chambered Structure.*—Present. *Membrane.*—Distinct, flexible, elastic. *Crista.*—Absent. *Terminal Finely Spiral Filament.*—Absent. *Flagella.*—Absent. *Highly Motile End Portion.*—Absent. *Division.*—Transverse. *Habitat of Genus.*—Free living in foraminiferous sand. *Other Species.*—*Nana*.

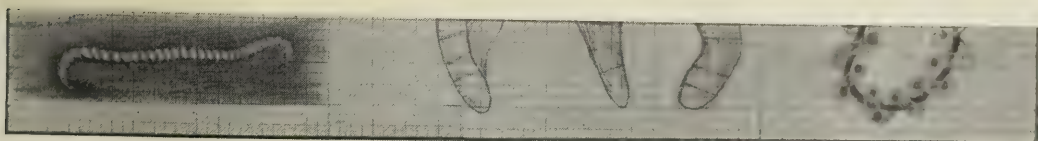
Genus.—*Cristispira* (Gross, 1910). *Type Organism.*—*Cristispira balbianii* (Certes, 1882) (Figs. 104 and 105). *Measurements.*—Length, 45 to 90 μ ; obtuse end. In stained preparations the end may be sharply pointed, but this is due

to shrinkage by fixing reagents. Diameter, 1 to 1.5 μ ; cylindrical. Waves, 2 to 5, sometimes more, large, irregular, shallow. In a dying specimen the waves may be more numerous and regular. *Axial Filament*.—Absent. *Chambered Structure*.—Present. *Membrane*.—Distinct, flexible, elastic. *Crista*.—Present, a ridge-like membrane. Spirally wound body. *Terminal Finely Spiral Filament*.—Absent. *Flagella*.—Absent. *Highly Motile End Portion*.—Absent. *Division*.—Transverse. *Habitat of Genus*.—Parasitic in the alimentary canals of shell-fish. *Other Species*.—*Ostræ*, *anodontæ*, *modiolæ*, *veneris*, *tapetos*, *chamæ*, etc. *Staining Prop-*



CORRECTION

On page 15, Vol. xxxi, 1919, the scale of Text-fig. 1 should read 0, 5, 10, and 15 μ instead of 0, 10, 20, and 30 μ .



TEXT-FIG. 1. Diagram contrasting the characteristic features and relative proportions of *Spironema*, *Treponema*, *Cristispira*, *Saprospira*, *Spirochaeta*, and *Leptospira*. The scale in microns is given in the upper left-hand corner of the figure.

erties of Axial Filament and Cell Membrane.—Membrane behaves like chitin or cutin substance. Stains violet by Giemsa's solution and light gray by iron-hematoxylin. *Staining Properties of Body*.—The body is alternately stained red and bluish violet and the crista red by Giemsa's solution. Iron-hematoxylin brings out sharp septa and a layer of chromatin granules. *Trypsin Digestion*.—Membrane resistant. Crista and chambers disappear. *Bile Salts (10 Per Cent)*.—Crista quickly destroyed. Body not attacked. *Saponin (10 Per Cent)*.—Crista becomes fibrillar, then indistinct. Body not affected.

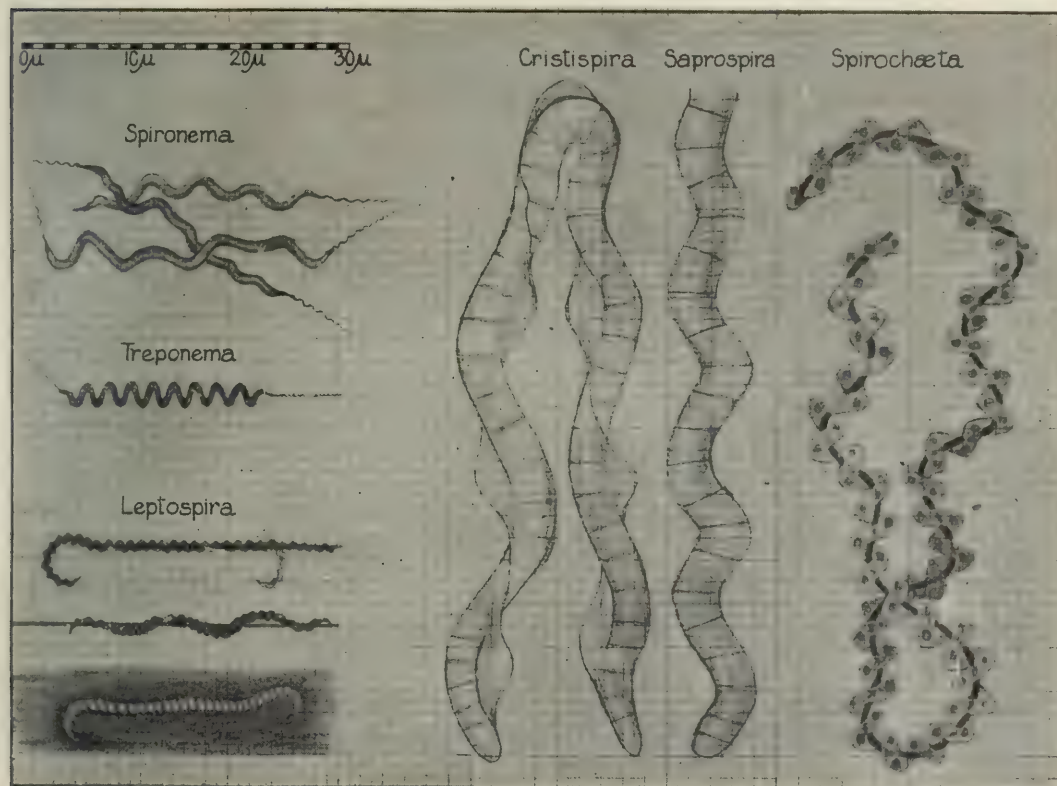
conditions the spirals of the spironemata acquire such rigidity and regularity that they, too, may be called treponemata. Dobell⁸ and Gross,⁶ independently of each other, and without any knowledge as to the morphological modifications due to cultivation, regarded the distinction between *Treponema* and *Spironema* as insufficient to maintain two separate genera, and Dobell chose the term *Treponema* and Gross *Spironema* for the same group of organisms. In my opinion the characteristics of *Treponema* and *Spironema*, under natural conditions, are sufficiently pronounced to justify retaining the two terms in classification. Neither *Treponema* nor *Spironema* has any feature which is likely to be confused with those of the other

of cutin-like substance. Stains violet by Gram's solution and grey by methematoxylin. *Staining Properties of Body*.—Plasmic spirals of the body stain with eosin, rubin, etc. Contain volutin granules. *Trypsin Digestion*.—Axial filament resistant. *Bile Salts (10 Per Cent)*.—Becomes shadowy pale but is not dissolved. *Saponin (10 Per Cent)*.—Lives 30 minutes. Later becomes shadowy, but is not dissolved.

Genus.—*Saprospira* (Gross, 1911). *Type Organism*.—*Saprospira grandis* (Gross, 1911). *Measurements*.—Length, 100 to 120 μ ; obtuse end. Diameter, ? μ ; cylindrical. Waves, large, inconstant, shallow, irregular, 3 to 5 in number. Sometimes almost straight. *Axial Filament*.—Absent. *Chambered Structure*.—Present. *Membrane*.—Distinct, flexible, elastic. *Crista*.—Absent. *Terminal Finely Spiral Filament*.—Absent. *Flagella*.—Absent. *Highly Motile End Portion*.—Absent. *Division*.—Transverse. *Habitat of Genus*.—Free living in foraminiferous sand. *Other Species*.—*Nana*.

Genus.—*Cristispira* (Gross, 1910). *Type Organism*.—*Cristispira balbianii* (Certes, 1882) (Figs. 104 and 105). *Measurements*.—Length, 45 to 90 μ ; obtuse end. In stained preparations the end may be sharply pointed, but this is due

to shrinkage by fixing reagents. Diameter, 1 to 1.5 μ ; cylindrical. Waves, 2 to 5, sometimes more, large, irregular, shallow. In a dying specimen the waves may be more numerous and regular. *Axial Filament*.—Absent. *Chambered Structure*.—Present. *Membrane*.—Distinct, flexible, elastic. *Crista*.—Present, a ridge-like membrane. Spirally wound body. *Terminal Finely Spiral Filament*.—Absent. *Flagella*.—Absent. *Highly Motile End Portion*.—Absent. *Division*.—Transverse. *Habitat of Genus*.—Parasitic in the alimentary canals of shell-fish. *Other Species*.—*Ostræ*, *anodontæ*, *modiolæ*, *veneris*, *tapetos*, *chamæ*, etc. *Staining Prop-*



TEXT-FIG. 1. Diagram contrasting the characteristic features and relative proportions of *Spirochæta*, *Saprospira*, *Cristispira*, *Leptospira*, and *Treponema*. The scale in microns is given in the upper left-hand corner of the figure.

Properties of Axial Filament and Cell Membrane.—Membrane behaves like chitin or cutin substance. Stains violet by Giemsa's solution and light gray by iron-hematoxylin. *Staining Properties of Body*.—The body is alternately stained red and bluish violet and the crista red by Giemsa's solution. Iron-hematoxylin brings out sharp septa and a layer of chromatin granules. *Trypsin Digestion*.—Membrane resistant. Crista and chambers disappear. *Bile Salts (10 Per Cent)*.—Crista quickly destroyed. Body not attacked. *Saponin (10 Per Cent)*.—Crista becomes fibrillar, then indistinct. Body not affected.

Genus.—*Spironema* (Vuillemin, 1905). *Type Organism.*—*Spironema recurrentis* (Lebert, 1874¹⁷) (Figs. 94 to 96). *Measurements.*—Length, 8 to 16 μ ; pointed ends. Diameter, 0.35 to 0.5 μ ; cylindrical or slightly flattened. Spirals, large, wavy, inconstant, about five in number. Closer and more regular in cultures. *Axial Filament.*—Probably present. *Chambered Structure.*—Absent. *Membrane.*—Delicate, flexible, double contoured. *Crista.*—Absent. *Terminal Finely Spiral Filament.*—Present, easily seen in cultures. *Flagella.*—Absent. *Highly Motile End Portion.*—Absent. *Division.*—Transverse, possibly also longitudinal. *Habitat of Genus.*—Numerous pathogenic and non-pathogenic varieties. *Other Species.*—*Carteri, kochi, novyi, duttoni, berbera, aegyptica, gallinarum, anserina, theileri, equi, muris, eugyratum, microgyratum, buccalis, refringens*, etc. *Staining Properties of Axial Filament and Cell Membrane.*—Membrane difficult to differentiate. *Staining Properties of Body.*—Stains violet by Giemsa's solution. *Bile Salts (10 Per Cent).*—Disintegration complete. *Saponin (10 Per Cent).*—Immobilized in 30 minutes then broken up in a few hours. In some there is an axial filament laid bare.

Genus.—*Treponema* (Schaudinn, 1905). *Type Organism.*—*Treponema pallidum* (Schaudinn and Hoffmann, 1905¹⁸) (Figs. 69 to 72 and 103). *Measurements.*—Length, 6 to 14 μ ; pointed ends. Diameter, 0.25 to 0.3 μ ; cylindrical. Spiral amplitude, 1 μ ; regular, rigid. Spiral depth, 0.8 to 1 μ ; very constant. Waves, one or more slight undulating curves may be present. *Axial Filament.*—Doubtful. The whole seems to consist of a spirally wound axial filament. *Chambered Structure.*—Absent. *Membrane.*—Doubtful; if there is one it must be flexible. *Crista.*—Absent. *Terminal Finely Spiral Filament.*—Present. Easily seen in cultures. *Flagella.*—Absent. *Highly Motile End Portion.*—Absent. *Division.*—Transverse or possibly also longitudinal. *Habitat of Genus.*—Two pathogenic and several harmless parasites. *Other Species.*—*Pertenue, microdentium, macrodentium, mucosum, calligyrum, minutum*. *Staining Properties of Axial Filament and Cell Membrane.*—Membrane not recognizable. *Staining Properties of Body.*—Stains pink by Giemsa's solution. *Trypsin Digestion.*—Resists digestion for many days. *Bile Salts (10 Per Cent).*—Disintegration complete. *Saponin (10 Per Cent).*—Broken up in time.

Genus.—*Leptospira* (Noguchi, 1917). *Type Organism.*—*Leptospira icterohæmorrhagiæ* (Inada and Ido, 1914) (Figs. 1 to 68, 101, and 102). *Measurements.*—Length, 7 to 9 to 14 μ ; exceptionally 30 to 40 μ ; pointed ends. Diameter, 0.25 to 0.3 μ ; cylindrical. Spiral amplitude, 0.45 to 0.5 μ ; regular, rigid. Spiral depth, 0.3 μ ; regular. Waves, one or more gentle wavy curves throughout the entire length. When in a free space, one or both ends may be semicircularly hooked, while in semisolid media the organism appears serpentine, waved, or bent. Its flexibility is most striking. *Axial Filament.*—Not recognized. *Chambered Struc-*

¹⁷ Lebert, H., Rückfallstyphus, Flecktyphus und Cholera, in von Ziemssen, H., Handbuch der speciellen Pathologie und Therapie, Leipsic, 1874, ii 267.

¹⁸ Schaudinn, F., and Hoffmann, E., *Arb. k. Gsndtsamte.*, 1905, xxii, 527.

ture.—Absent. Membrane.—Not recognized. Crista.—Absent. Terminal Finely Spiral Filament.—Not recognized. Flagella.—Absent. Highly Motile End Portion.—Well developed in the last six to eight spirals. Division.—Transverse. Habitat of Genus.—One pathogenic and one possibly non-pathogenic variety known. Other Species.—*Biflexa* (Wolbach and Binger). Staining Properties of Axial Filament and Cell Membrane.—Membrane not recognizable. Staining Properties of Body.—Stains reddish violet by Giemsa's solution. Bile Salts (10 Per Cent).—Easily dissolved. Saponin (10 Per Cent).—Completely resistant.

The comparative dimensions of these representative organisms may be shown by putting side by side the diameter, spiral amplitude, spiral depth, and length of each, taking the diameter of the finest member, *Leptospira icterohæmorrhagiæ*, as a unit of comparison (Table I).

TABLE I.

Organism.	Thickness.	Spiral amplitude.	Spiral depth.	Length.
<i>Leptospira icterohæmorrhagiæ</i> ...	1 (0.25 μ)	2 (0.5 μ)	1.2 (0.3 μ)	56 (14 μ)
<i>Treponema pallidum</i>	1.2 (0.3 μ)	4 (1 μ)	3.6 (0.9 μ)	48 (12 μ)
<i>Spironema obermeieri</i>	2 (0.5 μ)	12 (3 μ)	6 (1.5 μ)	32 (8 μ)
<i>Cristispira balbianii</i>	5 (1.2 μ)	60 (15 μ)	24 (6 μ)	200 (50 μ)
<i>Saprospira grandis</i>	5 (1.2 μ)	32 (8 μ)	8 (2 μ)	400 (100 μ)
<i>Spirochæta plicatilis</i>	3 (0.75 μ)	18 (4.5 μ)	6 (1.5 μ)	600 (150 μ)

One may obtain the comparative proportions for each genus by using the diameter of its representative member as a unit of comparison, as in Table II.

TABLE II.

Organism.	Thickness.	Spiral amplitude.	Spiral depth.	Length.
<i>Spirochæta plicatilis</i>	1 (0.75 μ)	6 (4.5 μ)	2 (1.5 μ)	200 (150 μ)
<i>Saprospira grandis</i>	1 (1.2 μ)	7 (8 μ)	1.8 (2 μ)	83 (100 μ)
<i>Cristispira balbianii</i>	1 (1.2 μ)	13 (15 μ)	5 (6 μ)	41 (50 μ)
<i>Spironema obermeieri</i>	1 (0.5 μ)	6 (3 μ)	3 (1.5 μ)	16 (8 μ)
<i>Treponema pallidum</i>	1 (0.3 μ)	3.3 (1 μ)	3 (0.9 μ)	40 (12 μ)
<i>Leptospira icterohæmorrhagiæ</i> ...	1 (0.25 μ)	2 (0.5 μ)	1 (0.25 μ)	56 (14 μ)

The proportions are distinctive for each genus, and form, with other differentiating features already discussed, a fairly well established basis for the classification of these spiral organisms, hitherto so indis-

criminated by the general name of spirochetes. It would be desirable, in describing a new spiral organism, to place it in one of the six classes discussed, since under the vague name of spirochete no one can visualize the actual features of the organism in question, while if it is called *Leptospira*, for example, certain definite features are connoted, and confusion with other so called spirochetes is avoided. This is particularly important when one is examining specimens of urine such as those from certain cases in which a *Leptospira* or a *Treponema* may be present alone or together, as in a study of trench infections. Patterson¹⁹ and Nankivell and Sundell²⁰ discovered the latter type in cases of trench fever of unknown origin, while the former has been found responsible for a number of cases of various trench affections.^{21, 22}

A brief note may be made of the relation of *Leptospira* to a comparatively minute species of spirochete, *Spirochæta stenostrepta*, described by Zuelzer⁷ (Figs. 109 and 110). The organism was found in stagnant water with *Spirochæta plicatilis*. It has a diameter of $0.25\ \mu$ and a length of 20 to 60 μ , seldom reaching a length of 200 μ . In a short specimen which measured 13 μ there were eleven spirals. In life an axial filament was recognized. Here the leptospira can be distinguished by its lack of an axial filament and its closer spirals. In the latter respect certain oscillatorial organisms such as *Spirulina vesicolor* (Figs. 111 and 112), or *Spirulina tenuissima* have a superficial resemblance to leptospira, but their multicellular structure, which can be demonstrated by subjecting them to a preliminary treatment with trypsin solution before staining, shows them to be very different. Each coil here represents an individual cell separated from the adjoining cells by walls. The spirulina has blunt ends and does not exhibit the active, brusque movements characteristic of leptospira.

¹⁹ Patterson, S. W., *J. Roy. Army Med. Corps*, 1917, xxix, 503.

²⁰ Nankivell, A. T., and Sundell, C. E., *Lancet*, 1917, ii, 672, 836.

²¹ Couvy, L., and Dujarric, R., *Compt. rend. Soc. biol.*, 1918, lxxxi, 22.

²² Dudgeon, L. S., *Lancet*, 1917, ii, 823.

SUMMARY.

The present study deals with the morphology and systematic position of the causative agent of infectious jaundice. There are several features which are not found in any of the hitherto known genera of Spirochætoidea which led me to give this organism an independent generic name, *Leptospira*, denoting the peculiar minute elementary spirals running throughout the body. The absence of a definite terminal flagellum or any flagella, and the remarkable flexibility of the terminal or caudal portion of the organism are other distinguishing features. Unlike all other so called spirochetes the present organism resists the destructive action of 10 per cent saponin.

A detailed comparative study of related genera, including *Spirochæta*, *Sapropsira*, *Cristispira*, *Spironema*, and *Treponema*, has been given with the view of bringing out more strongly the contrast between them and the new genus.

A study has been made to discover whether any differential features exist among the strains of *Leptospira icterohæmorrhagiæ* derived from the American, Japanese, and European sources, but none has been found.

It is hoped that the creation of a new genus may facilitate a more exact morphological description than has hitherto been possible, due to the vague use of the term *Spirochæta* which indiscriminately covered at least six large genera of spiral organisms.

EXPLANATION OF PLATES.

PLATE 25.

Figs. 1 to 23 show the morphological features of the American strain of *Leptospira icterohæmorrhagiæ* in stained preparations.

FIG. 1. *Leptospira icterohæmorrhagiæ* in the blood of an experimentally infected guinea pig, showing irregular refringent waves, but no minute elementary spirals. Methyl alcohol fixation and Giemsa's solution. $\times 1,000$.

FIG. 2. The same in a liver emulsion from a similar animal. Except for the few moderate undulations of the body, there is no indication here that these are spiral organisms. Methyl alcohol fixation and Giemsa's solution. $\times 1,000$.

FIG. 3. The same in a kidney emulsion. Fixation and staining the same as above. $\times 1,000$.

FIG. 4. The same in a blood specimen of an infected guinea pig. Fixation and staining the same as above. $\times 1,000$.

Figs. 1 to 4 are intended to show the appearance of the leptospiræ in an air-dried specimen, fixed with methyl alcohol, and stained with Giemsa's solution. They do not show any elementary spirals and appear as smooth, somewhat wavy filaments.

FIGS. 5 to 11. *Leptospira icterohæmorrhagiæ* in stained preparations from a culture in its first generation on the 5th day. They were fixed when moist by osmic acid vapor for 2 minutes, then hardened in absolute alcohol for 30 minutes, and after being thoroughly washed in distilled water, were stained over night with Giemsa's solution (1:20 dilution). In these preparations there were many instances where the fixation and staining were not so satisfactory as in the specimens shown in these photomicrographs. A careful examination makes possible recognition of the closely set, minute, regular spirals throughout the entire length of the organism. With a magnification of 1,000 they are almost too minute to enable one to count the number of the spirals. $\times 1,000$.

FIGS. 12 to 23. *Leptospira icterohæmorrhagiæ* magnified 3,000 times, which brings out the features more distinctly. All except Figs. 21 to 23 show the elementary spirals well. There are ten to twelve spirals to every 5μ , making the distance between the apex of one spiral to that of the next about 0.5μ . The terminal portions of the organisms are recognized by the gradually decreasing diameter and the coloration, which is lighter than that of the main portion of the body. These end portions seem to possess about six elementary spirals and measure about 3μ in length. They exhibit remarkable activity and flexibility and serve as propellers in progression in free space and as feelers in guiding the organism through a semisolid medium. Note Fig. 12.

Fig. 18 shows a specimen fixed probably during a somersault movement. The elementary spirals appear as dimly stained cross bars (imperfect fixation).

Fig. 21 shows three organisms attaching themselves to a red corpuscle. The spirals are not distinctly brought out, but one recognizes them as more intensely stained dots, arranged obliquely with respect to the optical axis of the organism.

Fig. 22 (also Figs. 13 and 19) shows a specimen fixed while rotating on its axis in a free space. The organism was otherwise stationary, as shown by its symmetrically bent hooks. Compare with Fig. 20, which has one hook, and therefore must be proceeding in the direction of the straight end.

The two specimens in Fig. 23 show no definite direction of progression. The spirals, though not well fixed, are fixed sufficiently for recognition.

In Figs. 12, 15, 17, 21, and 23, there is a clear space, or halo (about 0.15μ wide) about the organisms along the entire length. Whether this clear zone, or halo, indicates the presence of a less chromatic membrane enveloping the organisms or is merely due to the dispersion of particles (culture media) from their immediate neighborhood by their rotary movements cannot yet be determined.

PLATE 26.

FIGS. 24 to 35. Specimens of the American strain of *Leptospira icterohæmorrhagiæ* as seen under the dark-field microscope.

Fig. 24. The organisms in a liver emulsion of an experimentally infected guinea pig. They are in resting position and show no characteristic hooked ends. One isolated leptospira has both ends hooked, but not typically, as it would be while actively rotating or progressing in a free space. The spirals appear as regularly set cross bands. $\times 1,000$.

Figs. 25 and 26. A higher magnification of the same specimens. The finely set regular spirals are distinctly shown at the right in Fig. 24, and the cross-barred or dotted aspect of the spirals is shown in the other two of the same figure and also in Fig. 26. $\times 3,000$.

Fig. 27 ($\times 1,000$) and Figs. 28, 29, and 30 ($\times 3,000$) show the leptospiræ in the kidney emulsion of an infected guinea pig. Except for the specimen at the center of Fig. 27, the organisms are in undulatory positions, with gracefully wound, rather loose waves. This position almost always indicates that the organisms are in a semisolid medium, which they are penetrating by means of spiral propulsion. They often remain in the same position for some time before renewing their efforts to extricate themselves. Their dotted or cross-barred appearance remains unmodified under these circumstances.

Fig. 31 ($\times 1,000$) and Fig. 32 ($\times 3,000$) show similar but more pronounced characteristic features.

Fig. 34. The minute elementary spirals are plainly seen in the three entangled leptospiræ in the right upper corner, while in three organisms of Fig. 33 they are recognizable only as dots or bars. $\times 3,000$.

Fig. 35. A large mass of leptospiræ in a fluid culture 3 weeks old. They grow considerably longer in such a medium and form a mass of entangled organisms having the same minute elementary spirals as uncultivated specimens. $\times 1,000$.

PLATE 27.

FIGS. 36 to 57 represent the British strain (Stokes) of *Leptospira icterohæmorrhagiæ*.

FIG. 36. A Fontana preparation of the leptospiræ in the liver emulsion of an infected guinea pig. The elementary spirals can hardly be distinguished. $\times 1,000$.

FIG. 37. A badly fixed osmic acid-Giemsa preparation, in which one of the organisms on the extreme right appears as a negative image with minute elementary spirals well brought out. The dye settled about the leptospira without staining the organism itself. $\times 1,000$.

FIGS. 38 to 43. A preparation better fixed with osmic acid vapor and well stained with Giemsa's solution. The leptospiræ were cultivated 7 days at 28°C . In none of them is there any difficulty in discerning the individual elementary

spirals throughout the entire length of the organism. Perhaps owing to imperfect fixation, the elementary spirals in the terminal portions are less numerous and the spiral depth is shallower than in the main portion, which also takes on a more intense stain. In the majority of specimens the spiral amplitude of the main portion is about the same as that of the American or the Japanese strain ($0.5\ \mu$). There are a few specimens, however, which measure $0.6\ \mu$ from one spiral to the next. $\times 1,000$.

FIGS. 44 to 52. The same. $\times 3,000$.

FIGS. 53 to 57. Dark-field views of the leptospira. Figs. 53, 56, and 57 are from a fluid medium, and Figs. 54 and 55 from a semisolid medium. $\times 1,000$.

PLATE 28.

FIGS. 58 to 68. Dark-field views of the Japanese strain of *Leptospira icterohæmorrhagiæ* from a 7 day culture on semisolid medium. Figs. 58 to 62 are magnified 1,000 times and Figs. 63 to 68, 3,000 times. These photographs show the remarkable flexibility of the tight, elementary spirals of the organisms. The numerous circularly coiled specimens suggest the peculiar hoop-like coiling form of some specimens of *Cristispira balbianii* in the crystalline styles of oysters.

FIGS. 69 to 72. Dark-field views of *Treponema pallidum* which are given here for comparison with the leptospiræ. Their larger spiral amplitude and spiral depth, and their rigidity are sufficiently differentiating. Figs. 69 and 71 are magnified 1,000 times, and Figs. 70 and 72, 3,000 times.

PLATE 29.

Some of these photomicrographs are from stained and some from dark-field preparations. They are reproduced here to illustrate the differential characteristics of several constituent genera of the family of Spirochætoidea (Dobell). $\times 1,000$.

Treponema Group.

FIGS. 73 to 76. Dark-field views of a minute treponema (*Treponema minutum*, n. sp.) found in a smegma. Their average spiral amplitude is 0.9 to $1\ \mu$, spiral depth, 0.2 to $0.5\ \mu$, average number of spirals, eight to ten in 7 to $9\ \mu$, and thickness, $0.3\ \mu$.

FIGS. 80 to 83. *Treponema calligyrum* in smegma. Spiral amplitude, $1.75\ \mu$, depth, 0.5 to $1\ \mu$, four to seven spirals in 7 to $12\ \mu$, thickness, 0.4 to $0.5\ \mu$.

FIG. 84. The same from a culture.

FIG. 85. *Treponema microdentium* from the mouth.

FIG. 86. The same from a culture.

FIG. 88. *Treponema macrodentium* from a culture.

FIGS. 92 and 93. A treponema from the urine of a child, resembling the smallest smegma treponema (Figs. 73 to 76).

Spironema Group.

FIGS. 77 and 78. *Spironema refringens* from smegma. Spiral amplitude, 2 to 2.75 μ ; spiral depth, 0.5 to 1.5 μ ; four to eight spirals in 11 to 16 μ ; thickness, 0.7 μ .

FIG. 79. The same from a culture.

FIG. 87. *Spironema vincenti* from the mouth.

FIGS. 89 to 91. *Spironema buccalis* from the mouth. Spiral amplitude, 2.75 to 3.7 μ ; spiral depth, 0.7 to 1 μ ; four to seven and one-half spirals in 11 to 17 μ ; thickness, 0.5 to 1 μ .

FIGS. 94 and 96. *Spironema recurrentis* in a culture.

FIG. 95. The same in the blood of an infected mouse.

FIG. 97. *Spironema duttoni* in a culture.

FIG. 98. *Spironema kochi* in a culture.

FIG. 99. *Spironema gallinarum* in a culture.

FIG. 100. *Spironema novyi* in the blood of an infected rat.

Leptospira Group.

FIG. 101. *Leptospira icterohæmorrhagiæ*, American strain. $\times 1,000$.

FIG. 102. *Leptospira icterohæmorrhagiæ*, Japanese strain. $\times 1,000$.

FIG. 103. *Treponema pallidum* for comparison. Same magnification.

Cristispira Group.

FIG. 104. Dark-field view of *Cristispira balbianii* from oysters obtained near Woods Hole.

FIG. 105. The same. Osmic acid fixation. Stained with Giemsa's solution.

FIG. 106. *Cristispira veneris* (?) from clams obtained near Long Island Sound. Sublimite alcohol fixation and Heidenhain's iron-hematoxylin.

Saprospira Group.

FIG. 107. An organism possibly belonging to this genus. It was cultivated by me from oysters obtained near New York.

Spirochæta Group.

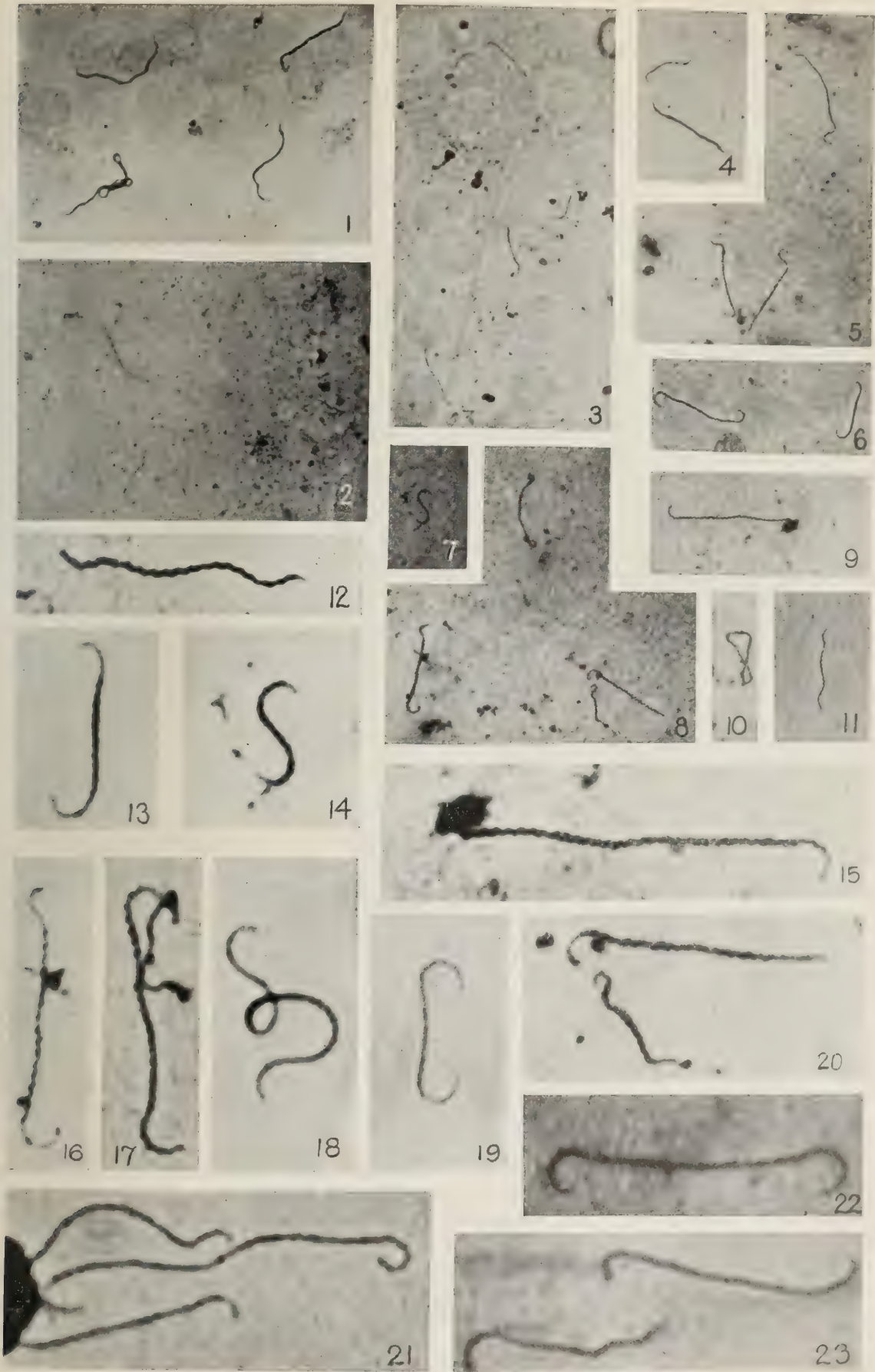
FIG. 108. *Spirochæta plicatilis*. Sublimite acetic-acid-alcohol fixation and iron-hematoxylin (after Zuelzer).

FIGS. 109 and 110. *Spirochæta stenostrepta* (after Zuelzer).

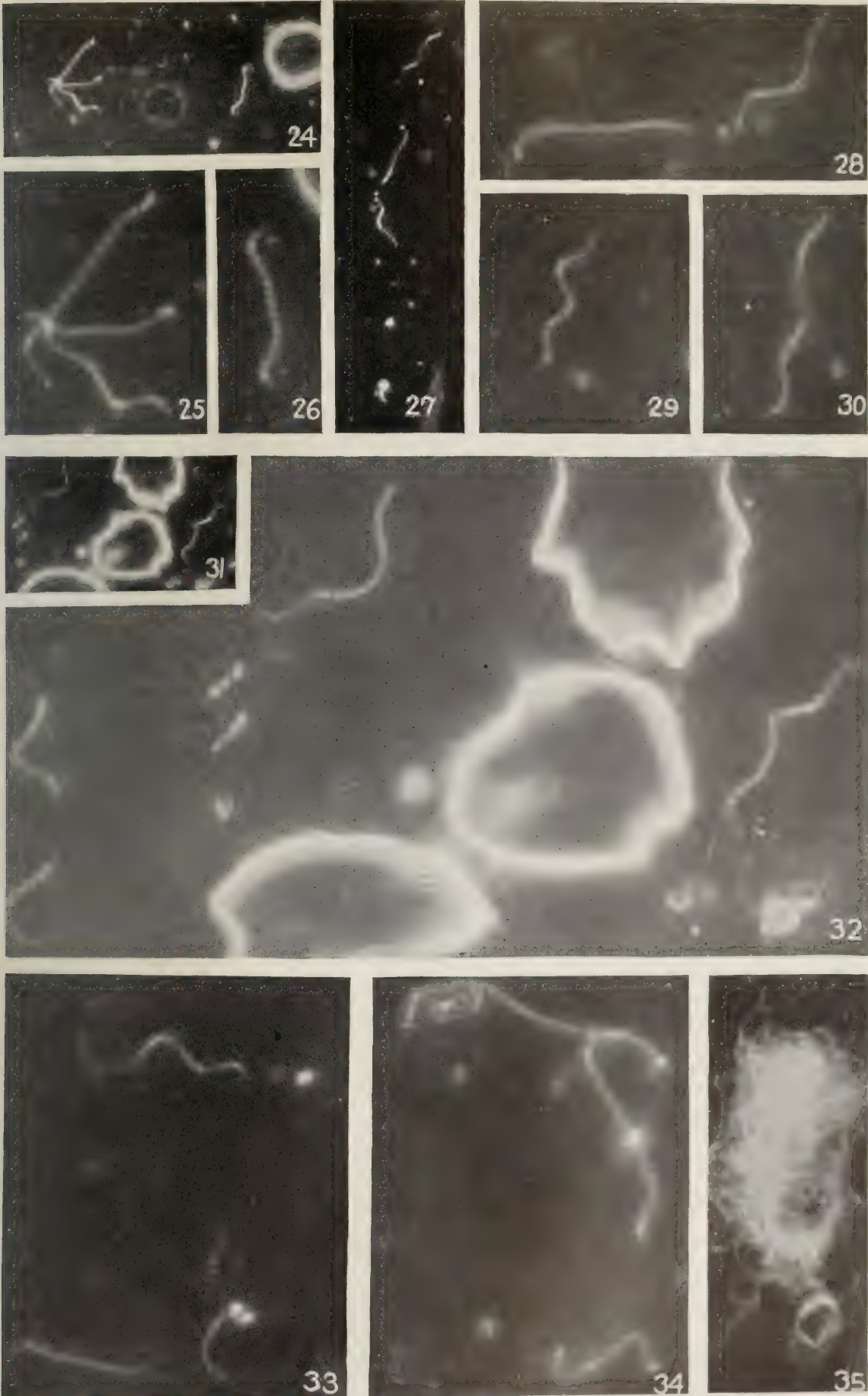
Spirulina Group.

FIG. 111. *Spirulina vesicolor*. This organism does not belong to the family of Spirochætoidea, but on account of its close spirals it is shown here. Iodine-alcohol and Delafield hematoxylin (after Zuelzer).

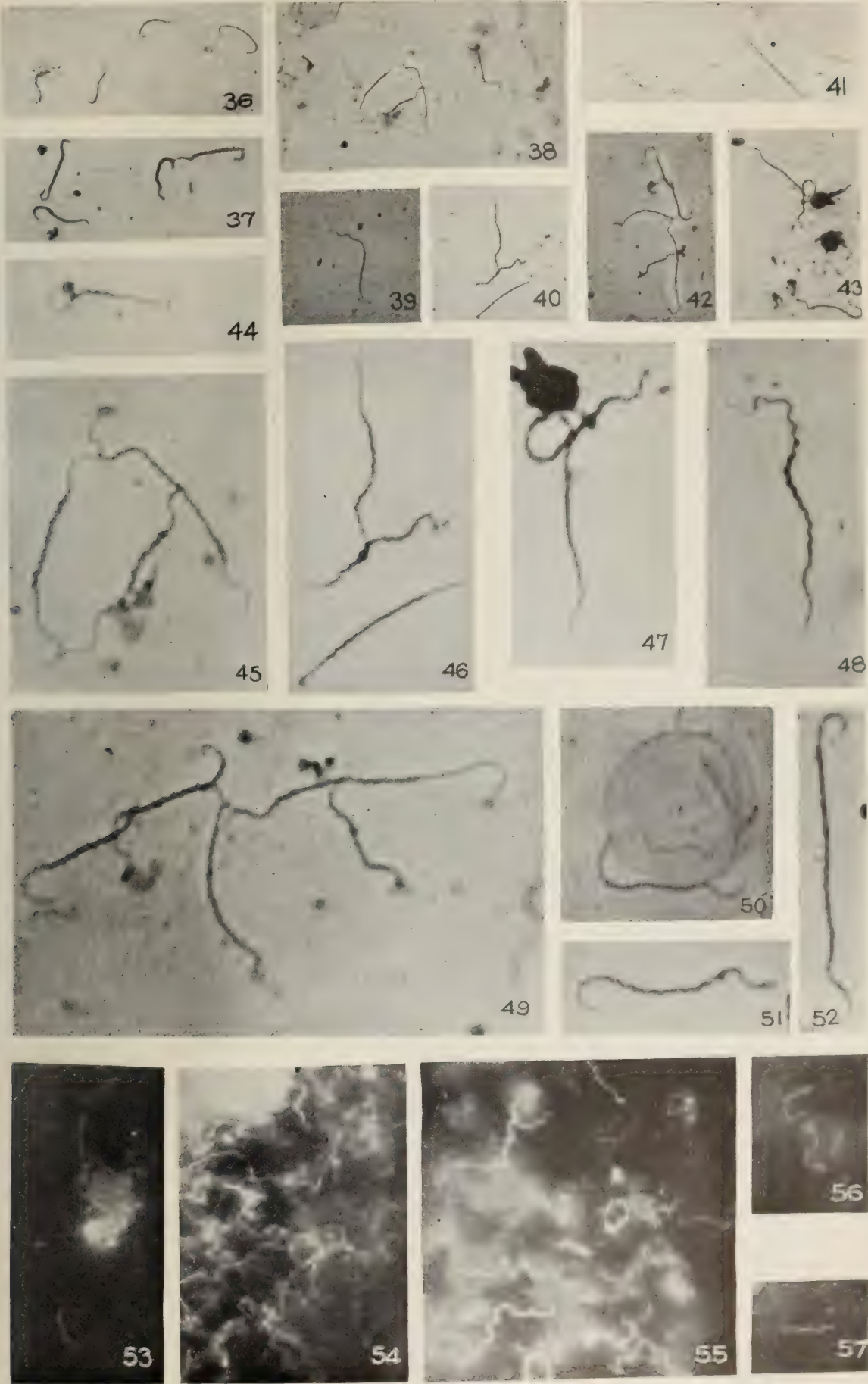
FIG. 112. The same, at another plane of focus, where the innermost structure is not brought out as in Fig. 111.



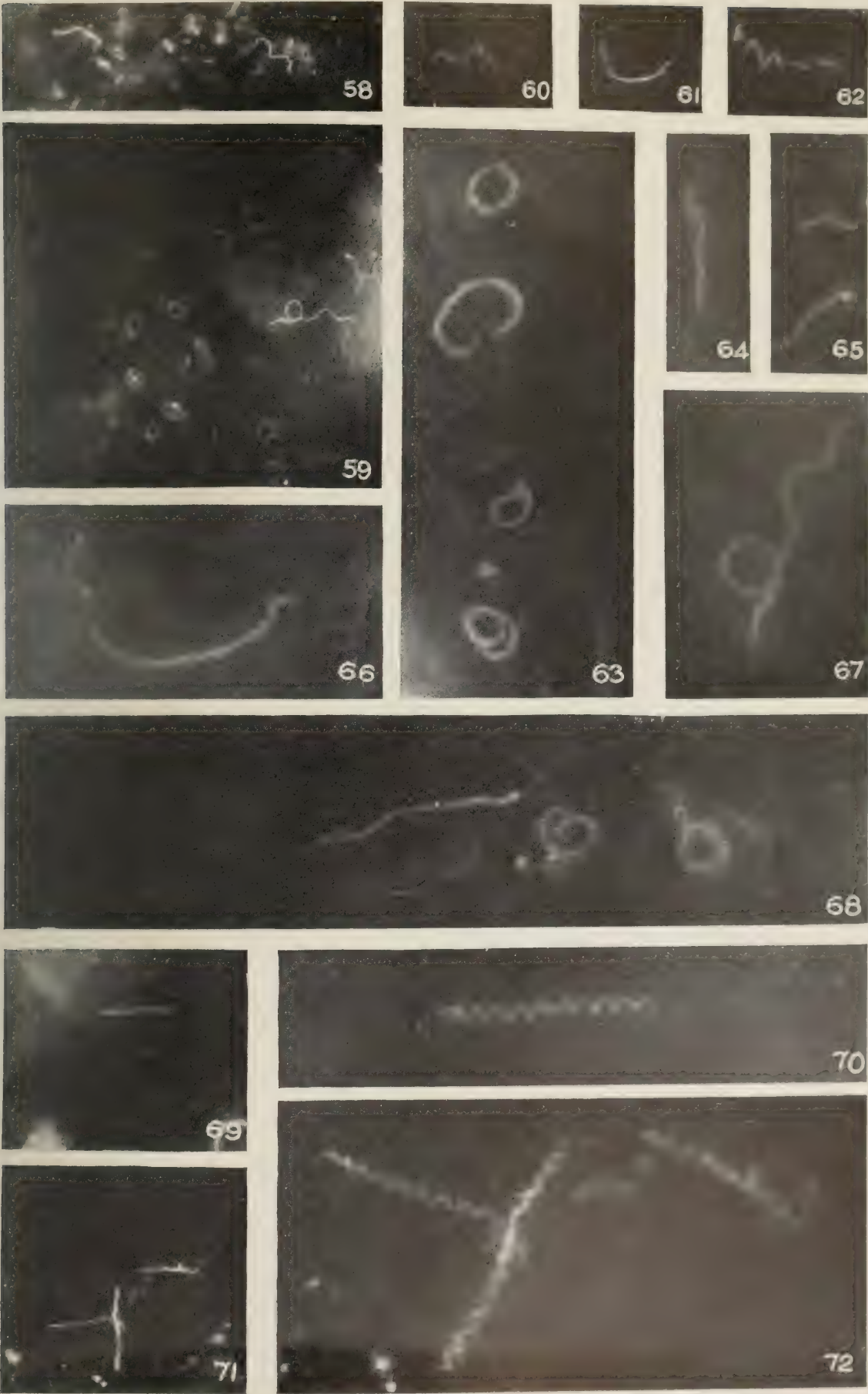
(Noguchi: Nomenclature of *Leptospira icterohemorrhagiae*.)



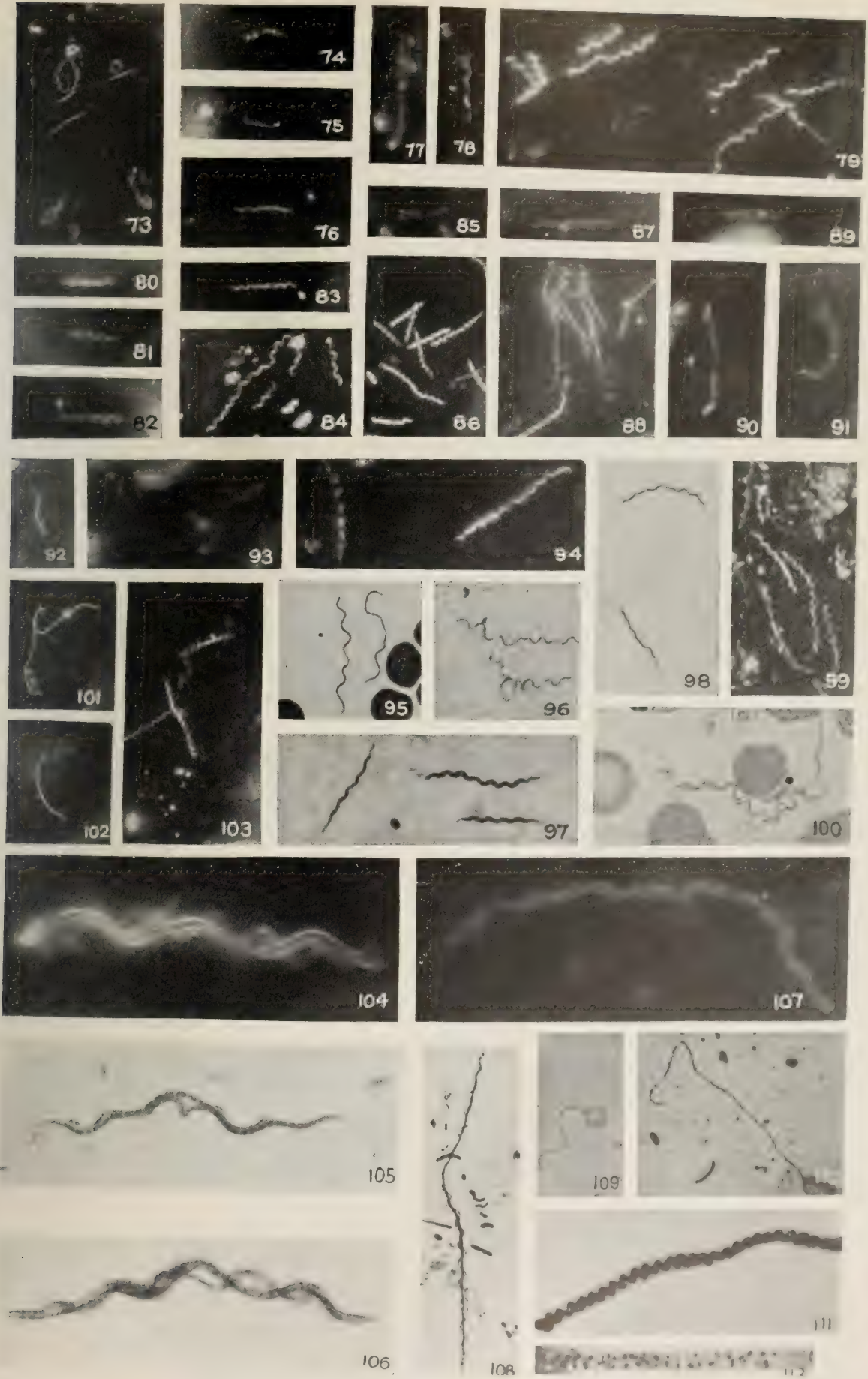
(Noguchi: Nomenclature of *Leptospira icterohamorrhagiae*.)



(Noguchi: Nomenclature of *Leptospira icterohamorrhagiae*.)



(Noguchi: Nomenclature of *Leptospira icterohamorrhagiae*.)



(Noguchi: Nomenclature of *Leptospira interrogans*.)

FURTHER STUDY ON THE CULTURAL CONDITIONS OF LEPTOSPIRA (SPIROCHÆTA) ICTEROHÆMORRHAGIÆ.

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The cultivation of *Leptospira* (*Spirochæta*) *icterohæmorrhagiæ*¹ is comparatively simple. It was first accomplished by Inada and his coworkers² by means of the method recommended by me for the cultivation of several varieties of blood spirochetes.³ Later, various techniques for the isolation of this organism on artificial media were proposed by Ito and Matsuzaki,⁴ Reiter,⁵ Martin, Pettit, and Vaudremer,⁶ and myself.⁷ While all the methods appear to have given satisfactory results, there is no unanimity as to the best one to be followed in routine work. As far as I am aware, there has been no critical analysis of the conditions requisite for uniform success in obtaining a culture. I wish to report here some of the results of my study of the various strains from Asiatic, European, and American sources.

Necessity of Fresh Serum Constituents for the Growth of Leptospira icterohæmorrhagiæ.

My first cultures of the Japanese, European, and American strains of *Leptospira icterohæmorrhagiæ* were obtained by employing a medium containing about 1 part of normal rabbit serum and 2 parts of Ringer's solution, with the addition of an adequate amount of citrate plasma.⁸ The rate of multiplication of the organism is faster at 37°C.

¹ Noguchi, H., *J. Exp. Med.*, 1918, xxvii, 575.

² Inada, R., Ido, Y., Hoki, R., Kaneko, R., and Ito, H., *J. Exp. Med.*, 1916, xxiii, 377.

³ Noguchi, *J. Exp. Med.*, 1912, xvi, 199.

⁴ Ito, T., and Matsuzaki, H., *J. Exp. Med.*, 1916, xxiii, 557.

⁵ Reiter, H., *Deutsch. med. Woch.*, 1916, xlii, 1282.

⁶ Martin, L., Pettit, A., and Vaudremer, A., *Compt. rend. Soc. biol.*, 1917, lxxx, 197.

⁷ Noguchi, *J. Exp. Med.*, 1917, xxv, 755.

⁸ About 0.5 part.

than at 25°C., but on the whole the first generation grows much more slowly than a later generation. It may be several days before growth is definitely ascertained.

The question may be raised as to what part of the serum is essential for the cultivation of the organism. For the purpose of determining this point, a portion of a mixture of rabbit serum 1 part, and Ringer's solution 3 parts, was heated to 60°C. for 30 minutes and another portion to 100°C. for 15 minutes. Unheated serum was used as control. It was found that heating to 100°C. for 15 minutes destroyed the nutrient value of the rabbit serum. Heating to 60°C. for 30 minutes reduced but did not destroy its cultural value as compared with the control. The nutrient principle of the serum, therefore, is closely associated with coagulable serum proteins. Filtration through the Berkefeld filter does not alter the cultural value of the serum medium.

Comparative Nutrient Value of Various Sera.

Not many animals are susceptible to the inoculation of *Leptospira icterohæmorrhagiæ*, and the guinea pig is the only animal in which the infection is almost invariably fatal. Rabbits are comparatively resistant, 1 to 2 cc. of a well growing pure culture being required to produce jaundice, whereas 0.000001 cc. of the same cultures may cause typical symptoms and death in a guinea pig. Dogs are more sensitive than rabbits, while cats, white rats, mice, and wild rats tolerate the infection and become carriers. A comparison of the suitability of various animal sera for purposes of cultivation of the organism is of practical as well as of biological interest.

Sheep Serum.—Of twelve different sheep sera, only four were found to be suitable, and in these the life of the organism was much shorter than in rabbit serum medium. A mixture of serum 1 part, Ringer's solution 3 parts, and 1.5 per cent agar 0.5 part was used. Undiluted sheep sera gave no better results, nor was the use of the citrate plasma from sheep advantageous.

Guinea Pig Serum.—Eight different lots of guinea pig sera were tested, each lot containing the sera from several animals, and good results were obtained in all. The sera were diluted three times with Ringer's solution and a small amount of agar or citrate plasma was

added. In this medium, however, the organism died out much sooner than in the rabbit serum medium.

Horse Serum.—Two out of four different horse sera proved to be very satisfactory, especially when used in a mixture of 1 part serum, 3 parts Ringer's solution, and 0.5 part 1.5 per cent agar. In this medium the culture survived for many weeks.

Calf Serum.—Only two calf sera were tested, but both gave a fairly good growth. A 1:4 dilution of serum with Ringer's solution was better than the undiluted serum. Martin, Pettit, and Vaudremer⁶ recommend a 1:10 dilution of this serum as most suitable.

Goat Serum.—The only serum tested was very suitable when used in a mixture of 1 part serum, 3 parts Ringer's solution, and 0.5 part 1.5 per cent agar. The undiluted serum did not give so good a growth.

Donkey Serum.—The one available specimen proved totally unsuitable.

Pig Serum.—Two pig sera were tested, but the culture failed to grow in any concentration.

Rat Serum.—The sera from about twenty white rats were mixed and tested for their nutrient value. Diluted as well as undiluted sera were employed, but the results were negative.

Human Serum.—Five specimens which had been collected many months previous to the time of testing proved to be without any nutrient value for the organism in question. Two other specimens, which were freshly collected⁹ from syphilitic patients, were found to be fairly suitable when used in proportions of 1:1 and 1:3 with Ringer's solution. The culture was short lived, however, reaching its greatest growth in about 11 days at 37°C. and dying off during the following week. The growth of the culture in the rabbit serum control medium was still increasing when the other cultures died.

Ascitic Fluid.—Twenty different samples of ascitic fluid were tested. They were used undiluted and also in different dilutions with Ringer's solution, but up to the present time none has been found suitable for the cultivation of *Leptospira icterohæmorrhagica*.

These specimens were obtained through the courtesy of Dr. David J. Kaliski.

Nutrient Value of Organ Emulsions.

In the later stages of infection *Leptospira icterohæmorrhagiæ* invades the visceral organs in enormous numbers, the liver and kidneys being principally involved. One might infer, therefore, that these organs contain an abundant quantity of the substances favorable for the life and multiplication of the organism, and that an emulsion of these organs would constitute an ideal culture medium. The experimental data, however, did not support this assumption.

Emulsions of approximately 5 per cent in Ringer's solution were prepared with the liver, kidney, spleen, heart muscle, and testicle of a normal rabbit and a normal guinea pig, killed by bleeding, and tested for their nutrient value as culture media. In order to make the conditions of the media as varied as possible, the emulsions were used in four different ways: in one set of tubes the emulsion was used alone and unheated, in the second it was heated to 60°C., in the third it was heated to 100°C., and in the fourth there was added agar amounting to 0.3 per cent. The mixture of rabbit serum, Ringer's solution, and citrate plasma and that of rabbit serum, Ringer's solution, and agar were used as control media.⁷ In the media containing the organ emulsions no sign of growth of the spirochete was observed, while excellent cultures were obtained in the control media.

The organs of guinea pigs were just as unsuitable for the cultivation of the organism as those of rabbits. I was not unaware of the possible change in the reaction due to autolysis of the organ cells, or of the injurious effect which certain autolytic cleavage products might have, but the emulsions showed a weak alkaline reaction throughout the experiments.

When rabbit serum, in the proportion of approximately 25 per cent, was added to a number of the tubes containing the emulsions, the spirochete multiplied vigorously; therefore, the fact that no culture was obtained with pure organ emulsions must have been due to the absence of suitable nutrient substances for the organism.

Egg White and Egg Yolk as Culture Media.

The failure of various organ emulsions to serve as culture media turned my attention to the possibility of utilizing egg white and egg

yolk for the purpose. The white and yolk of an egg were separated and each was diluted with Ringer's solution in different proportions: 2.5 cc. + Ringer's solution 2.5 cc.; 1 cc. + Ringer's solution 4 cc.; 0.5 cc. + Ringer's solution 4.5 cc.; and 0.25 cc. + Ringer's solution 4.75 cc. In each instance one set of tubes was used in the fresh state and the other heated to 55°C. for 24 hours with a view to possible improvement of nutrient value. In none of the egg media was any culture obtained, nor did the addition of the rabbit serum enhance their nutrient value beyond that of the serum.

Concentration of the Serum in Culture Media.

The importance of the presence of serum for the successful growth of the spirochete having been demonstrated, the following experiments were undertaken in order to determine the influence of various

TABLE I.

Japanese strain.	37°C.		26°C.	
	7 days.	30 days.	7 days.	30 days.
Undiluted rabbit serum.....	+	+++	+	+++
50 per cent rabbit serum + Ringer's solution....	+	+++	+	+++
33 " " " " + " "	+	+++	+	+++
25 " " " " + " "	+	+++	+	+++
20 " " " " + " "	+	+++	+	+++
15 " " " " + " "	+	+++	+	+++
10 " " " " + " "	+	+++	++	+++
5 " " " " + " "	+	+	+	+++

European strain.	Cultures, 30 days at 26°C.	Proteins precipitable with 10 volumes of absolute alcohol.
33 per cent rabbit serum + Ringer's solution	+++	Copious coarse precipitate and opalescence.
20 " " " " + " "	++	Copious coarse precipitate and opalescence.
10 " " " " + " "	++	Minute granules and opal- escence.
5 " " " " + " "	+	Opalescence.
2 " " " " + " "	—	"
1 " " " " + " "	—	Granular.
0.5 " " " " + " "	—	

TABLE I—*Concluded.*

American strains.								Cultures, 30 days at 26°C.		
								Strain 1	Strain 2	Strain 3
33	per	cent	rabbit	serum	+	Ringer's	solution...	+++	+++	+++
20	"	"	"	"	+	"	" ...	+++	+++	+++
10	"	"	"	"	+	"	" ...	++	+++	++
5	"	"	"	"	+	"	" ...	+	++	+
2	"	"	"	"	+	"	" ...	—	—	—
1	"	"	"	"	+	"	" ...	—	—	—
0.5	"	"	"	"	+	"	" ...	—	—	—

The above experiments show that a maximum growth may be obtained with all strains tested in a medium containing more than 20 per cent serum, while a 10 per cent serum medium may give as much growth, but only with certain strains. The growth is scanty in a 5 per cent serum solution, and in a medium containing 2 per cent or less there is no growth.

concentrations of serum upon the culture. Table I summarizes the results.

Influence of Diluents and of Salt Concentration upon the Culture.

The apparent indifference of the spirochete to salt constituents of the culture media was noticed from the beginning of the cultivation

TABLE II.

American strain No. 1	Cultures 30 days at 26° C.
Rabbit serum 1 cc. + 10 per cent sodium chloride 4 cc. = 8 per cent sodium chloride.....	+++
Rabbit serum 1 cc. + 10 per cent sodium chloride 2 cc. + water 2 cc. = 4 per cent sodium chloride.....	+++
Rabbit serum 1 cc. + 10 per cent sodium chloride 1 cc. + water 3 cc. = 2 per cent sodium chloride.....	+++
Rabbit serum 1 cc. + 10 per cent sodium chloride 0.5 cc. + water 3.5 cc. = 1 per cent sodium chloride.....	+++
Rabbit serum 1 cc. + 10 per cent sodium chloride 0.25 cc. + water 3.75 cc. = 0.5 per cent sodium chloride.....	+++
Rabbit serum 1 cc. + water 4 cc. = salt-free control.....	+++
" " 1 " + Ringer's solution 4 cc. = serum-Ringer's solution control.....	+++

experiments. Instead of Ringer's solution, a 0.9 per cent saline solution or distilled water could be used as a diluent. In fact sewer water and stagnant or ordinary tap water were found to be satisfactory diluents when previously rendered sterile by filtration or autoclaving. The organism displays great tolerance not only to various neutral salts or organic matter which are apt to be present in sewer or stagnant water, but also to an increasing concentration of sodium chloride. The relation of salt concentration to growth is shown in Table II.

There was no perceptible difference in the degree of growth of the organism in this experiment, or in its morphological features. The tonicity of the culture medium is apparently an unimportant factor.

Effect of Reaction upon the Culture.

Leptospira icterohæmorrhagiæ seems to be one of the most sensitive of the microorganisms to the reaction of the culture medium. A slight variation to acid or alkaline from a given optimum zone renders a medium totally unsuitable for the growth of the organism (Table III).

Considering the minuteness of the quantities of hydrochloric acid or sodium hydroxide which were added in these experiments, and the extent to which the reagents were finally diluted with serum and distilled water, one cannot fail to realize the great importance which the reaction of the culture medium must have in relation to the growth of the organism. Similar results were obtained with the Japanese and European strains. The first requisite to the successful cultivation of *Leptospira icterohæmorrhagiæ* appears to be an optimum reaction of the culture medium, which, in my experience, lies between a slight alkaline reaction and that resulting from subsequent multiple dilutions with indifferent diluents (distilled water, isotonic salt solution, Ringer's solution, etc.).

A considerable fluctuation was found by titration of the sera of several domestic animals. For example, 2 cc. of the sera of the sheep, donkey, ox, and pig, each mixed with 3 cc. of distilled water, required 0.4 cc. of 0.1 N hydrochloric acid to bring about a neutral reaction, and 0.6 cc. to cause distinct acidity and turbidity. Rabbit serum had a uniformly weaker reaction, only 0.2 cc. of 0.1 N hydro-

TABLE III.

American strain No. 1	Physical changes.	Reaction to litmus paper.	Result of cultivation at 30° C. for.	
			6 days.	18 days.
Rabbit serum 1 cc. + water 3 cc.	Clear.	Slight alkaline.	+++	++
“ “ 1 “ + 1.5 per cent agar 0.5 cc.	“	Slight alkaline.	+++	++++
Addition of acid.				
Rabbit serum 1 cc. + 0.1 N hydrochloric acid 0.1 cc.	Slight opalescence.	Neutral.	+	—
Rabbit serum 1 cc. + 0.1 N hydrochloric acid 0.1 cc. + 1.5 per cent agar 0.5 cc.	Slight opalescence.	“	+	—
Rabbit serum 1 cc. + 0.1 N hydrochloric acid 0.2 cc.	Many sandy precipitates on wall and bottom.	Trace of acid.	—	—
Rabbit serum 1 cc. + 0.1 N hydrochloric acid 0.2 cc. + 1.5 per cent agar 0.5 cc.	Slight opalescence.	Trace of acid.	+	—
Addition of alkali.				
Rabbit serum 1 cc. + 0.1 N sodium hydroxide 0.1 cc.	Clear.	Distinct alkaline.	—	—
Rabbit serum 1 cc. + 0.1 N sodium hydroxide 0.1 cc. + 1.5 per cent agar 0.5 cc.	“	Distinct alkaline.	—	—
Rabbit serum 1 cc. + 0.1 N sodium hydroxide 0.2 cc.	“	Stronger alkaline.	—	—
Rabbit serum 1 cc. + 0.1 N sodium hydroxide 0.2 cc. + 1.5 per cent agar 0.5 cc.	“	Stronger alkaline.	—	—

chloric acid being required to produce a neutral, and 0.35 cc. an acid reaction. The reaction of horse serum lay between that of the rabbit and that of the other animals. The fact that some of the latter sera showed a better nutrient value in dilution may be explained by the reduction of native alkalinity through dilution.

Oxygen Requirement of the Culture.

At the beginning of these cultivation experiments, I supposed *Leptospira icterohæmorrhagiæ* to be an obligatory or facultative anaerobe, because of its great facility for invading organs and multiplying in them. All attempts at cultivation failed as long as cultural conditions were employed which were calculated to produce anaerobiosis. The combination of conditions which I designated as aerotropic anaerobiosis¹⁰ several years ago, and which was successfully used for the cultivation of the relapsing fever spirochetes, gave fairly good results when a suitable serum was used. But in the tubes to which a piece of fresh rabbit kidney was added, the cultures grew less luxuriantly and died out sooner than in the control tubes without the tissue. The simplicity of the cultural requirements of this organism was a surprise and led to the inference that the organism is an aerobe. When a number of subcultures of the Japanese, European, and American strains were cultivated at 37°C. in an anaerobic apparatus and another set without the exclusion of oxygen, excellent growth took place in all tubes where oxygen was accessible, while not a single organism could be found in the tubes kept in an anaerobic apparatus. The tubes were taken out of the anaerobic jar after 12 days and allowed to stand for several days at 37°C., but no new culture developed, probably because of the death of the organism during its stay in the anaerobic apparatus. *Leptospira icterohæmorrhagiæ*, therefore, has been shown to be an obligatory aerobe.

Detrimental Conditions Caused by Physical Hindrances to the Penetration of Oxygen into the Medium.

For obligatory aerobic bacteria a slant or plate agar or broth should be satisfactory, because most of this class of organisms grow in more

¹⁰Noguchi, *The Harvey Lectures*, 1915-16, 236.

or less discrete, often thick or elevated colonies on the surface of a solid medium. In broth the growth may be diffuse or superficial, forming a pellicle or thick scum. The use of a high layer agar or gelatin for the cultivation of such organisms means a waste of medium, since oxygen cannot penetrate the greater part of it. Since *Leptospira icterohæmorrhagiæ* is an obligatory aerobe, it follows that the addition of solid substances such as agar or gelatin, which must necessarily interfere with the entrance of oxygen into the medium, will be detrimental to the growth of the organism. The denser the concentration of agar or gelatin, the narrower is the zone to which oxygen can penetrate. The experiment summarized in Table IV shows the effect of different concentrations of agar or gelatin upon the culture. The gelatin and agar were made in a 0.5 per cent saline solution and adjusted to a slightly alkaline reaction.

TABLE IV.

Medium.	37°C.			26°C.		
	4 days.	7 days.	28 days.	4 days.	7 days.	28 days.
Gelatin (10 per cent) 4 cc.	—	—	—	—	—	—
{ Rabbit serum 1 cc. Gelatin (10 per cent) 3 cc.	+	—	—	<<+	—	—
{ Rabbit serum 1 cc. Gelatin (10 per cent) 1 cc. Ringer's solution 2 cc.	+	++++	++++	<+	+	++++
{ Rabbit serum 1 cc. Gelatin (10 per cent) 0.5 cc. Ringer's solution 2.5 cc.	+	++++	++++	<+	<+	++++
Agar (2 per cent) 4 cc.	—	—	—	—	—	—
{ Rabbit serum 1 cc. Agar (2 per cent) 3 cc.	+	++	<+	<<+	+	<<+
{ Rabbit serum 1 cc. Agar (2 per cent) 1 cc. Ringer's solution 2 "	+	++++	++++	+	++	++++
{ Rabbit serum 1 cc. Agar (2 per cent) 0.5 cc. Ringer's solution 2.5 "	++	++++	++++	+	++	++++

The experiment demonstrates the disturbing effect of gelatin when present in more than 7.5 per cent and of agar in more than 1.5 per cent. Agar, when added in proportions of 0.5 per cent and 0.25 per cent, considerably improved the cultural conditions. In this concentration it does not perceptibly hinder the penetration of oxygen into the medium and it offers to the spirochetes an ideal semisolid permeable substance. In this respect this particular culture medium is even better than a pure fluid medium. Gelatin, when added in proportions of 2.5 and 1.25 per cent, seems to have been neither beneficial nor detrimental to the growth of the culture.

Ordinary Culture Media and Leptospira icterohæmorrhagiæ.

It would be an economic advantage if a simpler method for the cultivation of this spirochete was devised. No culture was obtained, however, with any of the ordinary media, such as plain and 2 per cent glucose bouillon, Hiss serum water, litmus milk, plain and 2 per cent glucose agar, Loeffler's serum, glycerolated bouillon, and agar. A special bouillon medium formulated by Dr. Kligler was tried—1 per cent peptone, 0.5 per cent sodium phosphate, 0.1 per cent glucose, and 0.5 per cent sodium chloride—but without success. The presence of peptone, broth, casein, glucose, etc., instead of having a nutrient value for *Leptospira icterohæmorrhagiæ* in a suitable medium such as one containing the necessary amount of rabbit serum, seems to have a definite unfavorable influence upon the culture. The addition of a 10 per cent neutral solution of peptone 4.5 cc., to rabbit serum 1.5 cc., rendered the mixture unsuitable for a culture medium, as is not the case with indifferent diluents such as Ringer's solution, distilled water, or isotonic salt solution. Even the addition of approximately 1.5 per cent peptone suppressed growth to a marked degree. Bouillon or glucose bouillon are not good diluents for making up a culture medium for this organism.

Addition of Carbohydrates to Culture Media.

Akatsu,¹¹ while working in my laboratory, studied the action of various spirochetes upon many carbohydrates, but he did not find

¹¹ Akatsu, S., *J. Exp. Med.*, 1917, xxv, 375

definite fermentation phenomena in any of the organisms examined. With *Treponema mucosum* and *Treponema microdentium* a definite increase in the amount of acid was noticed. In the present experiment, the Japanese strain of *Leptospira icterohæmorrhagiæ* was cultivated in two sets of media of fourteen tubes each. In one set the media were made up of 1.5 cc. of rabbit serum, 1 cc. of a 10 per cent solution of carbohydrate, previously sterilized by filtration, 2.5 cc. of Ringer's solution, and 1 cc. of citrate plasma of the rabbit. In the other set 1 cc. of 2 per cent agar (melted) was used instead of the citrate plasma. The fourteen carbohydrates used in both sets were glucose, lactose, maltose, levulose, galactose, saccharose, dextrin, inulin, mannite, dulcitol, isodulcitol, arabinose, raffinose, and salicin. Tubes without carbohydrate and tubes also which were not inoculated with culture were used as controls.

The new generation of the culture became recognizable within a fortnight at 29°C. by the hazy layer at the top of the columns of culture media. The haze extended downwards from the surface to a depth of 1 to 1.5 cm. By examination under the dark-field microscope, the haze was found to represent dense diffuse colonies of actively multiplying spirochetes. The appearance of the haze was the same in the tubes containing the various carbohydrates as in the sugar-free control tubes. In the set where 1 per cent citrate plasma was used to form loose fibrin, the haze was less distinct but extended as far as 3 or 4 cm. below the surface, and the lower border was not sharply outlined as in the media with semisolid agar. The viability of the spirochetes was as great in the media containing carbohydrates as in those without carbohydrates. The reaction of the cultures failed to indicate any attack by the organism upon the carbohydrates. The reaction remained slightly alkaline to litmus paper as before cultivation, and was entirely comparable with the reaction in the spirochete-free controls.

Special attention was given to the detection of possible morphological modifications in the organisms grown in the presence of the carbohydrates, but none was recognized.

Influence of Temperature upon Cultivation.

Inada and his coworkers² found that *Leptospira icterohæmorrhagiæ* grows very well at room temperature, as it does at any temperature up to 37°C., but that at lower temperatures (20–25°C.) the organism survives longer than at 37°C.

I have cultivated three different strains of the spirochete at different temperatures. The results, as recorded in Table V, are self-explanatory. The media used consisted of rabbit serum 1 cc. + Ringer's solution 3 cc. + citrate plasma 1 cc. or 1.5 per cent agar 1 cc.

TABLE V.

Strains.	42°C.		37°C.		30°C.		25°C.		10°C.	
	7 days.	28 days.	7 days.	28 days.	7 days.	28 days.	7 days.	28 days.	7 days.	28 days.
Japanese.										
Plasma.	—	—	++++	++++	++++	++++	++++	++	++++	++
Agar.	—	—	+++	++++	+++	++++	+	<+	+	<+
European.										
Plasma.	—	—	++	+++	++	++	+	—	+	—
Agar.	—	—	++	+++	++++	++++	+	++	<<+	—
American										
No. 1.										
Plasma.	—	—	++	+++	++	+++	+	++	+++	++
Agar.	—	—	++	+++	++	++++	++	+++	++	+

The ability of the organism to multiply and remain active a long time at 10°C. is interesting from the epidemiological standpoint. It suggests that certain insects might serve as reservoirs of the virus.

Culture Media Recommended for Leptospira icterohæmorrhagiæ.

As a result of the experiments recorded on the relative nutrient value of various sera, the influence of reaction, oxygen tension, diluents, salts, and various other substances, I have formulated the following media:

A. Rabbit serum.....	1.5 parts.	
Ringer's solution.....	4.5 "	
Citrate plasma.....	1.0 part.	
Paraffin oil to cover the surface.		
B. Rabbit serum.....	1.5 parts.	
Ringer's solution.....	4.5 "	
2 per cent agar.....	1.0 part.	
Paraffin oil to cover the surface.		
C. Rabbit serum.....	1.5 parts.	} Semisolid portion.
Ringer's solution.....	4.5 "	
2 per cent agar.....	1.0 part.	
After solidification add:		
Rabbit serum.....	1.5 parts.	} Fluid portion.
Ringer's solution.....	4.5 "	
Paraffin oil to cover the surface.		

Growth usually begins much sooner in Medium A than in Medium B, but after a month more spirochetes will be found in B. For keeping up subcultures of various strains, Media A and B were simultaneously used in small test-tubes each containing 7 cc. of the composite medium.

For obtaining a large amount of culture, long necked flasks of medium capacity (50 to 100 cc.) were used. It was found best to fill the flasks with the medium to one-half or one-third their capacity and then to cover the surface with a very thin layer of paraffin oil. If the flasks are filled higher than this, oxygen becomes less accessible to the deeper part of the medium, especially when it contains agar. The use of a low layer semifluid medium (B) is based upon the fact, previously mentioned, that unrestricted multiplication of *Leptospira icterohæmorrhagiæ* takes place in such a medium on the surface stratum of 1 to 2 cm. Medium A is similarly semifluid, but the fibrin mass loosens and breaks up in time, especially by repeated withdrawal of the culture with pipettes, rendering the penetration of oxygen almost as easy as in a fluid medium. The flasks containing Medium A may therefore be filled half or two-thirds full, with a thin layer of paraffin oil.

Medium C seems to combine the advantages of Media A and B, the lower stratum being composed of Medium B, upon which, after solidification, is superimposed a mixture of rabbit serum and Ringer's solution (1:3). The medium is then inoculated and covered with a thin layer of paraffin oil. For subcultures, 0.1 or 0.2 cc. of a vig-

orously growing culture is pipetted on the surface of new culture media and then covered with paraffin oil.

D is a medium for acclimated strains. A fluid medium consisting of 1 part of horse or sheep serum and 3 parts of Ringer's solution or salt solution proved to be fairly suitable for strains which had become accustomed to the various media (A, B, C) during a period of several months.

SUMMARY AND CONCLUSIONS.

1. The presence of suitable animal or human serum is essential for the cultivation of *Leptospira icterohæmorrhagiæ*.

2. The nutrient value of serum is considerably reduced by heating to 60°C. for 30 minutes and is destroyed by boiling (100°C). Filtration through a Berkefeld filter does not diminish the nutrient value of the serum.

3. The cultural value of different animal sera varies considerably. It is entirely absent from the sera of the rat and the pig. The sera of the rabbit, horse, and goat are better suited for the growth of the organism than those of the guinea pig, sheep, donkey, or calf. Human serum is suitable, but not ascitic fluid.

4. Fresh or heated emulsions of the liver, kidney, heart muscle, or testicle of the normal guinea pig or rabbit have no cultural value for the organism. The same may be said of both the white and yolk of the hen's egg.

5. A luxuriant growth takes place in a medium of Ringer's solution to which more than 10 per cent of normal rabbit serum is added. There is only moderate growth with 5 per cent of serum, and none when less than 2 per cent is present. The use of an undiluted serum offers no advantage over a diluted one, provided the latter contains at least 10 per cent of serum. In the case of certain animal sera dilution seems to make them more suitable for cultivation purposes, owing perhaps to its reduction of their inherent alkalinity.

6. The tonicity of the culture medium has but little influence upon the growth and morphology of the organism. A medium containing distilled water as diluent or one containing 8 per cent sodium chloride seems to give identical results. The viability of the organism was

greatest in a medium in which Ringer's solution or isotonic salt solution was used as diluent.

7. The reaction of the medium is an important factor in the cultivation of the organism, which thrives most vigorously in a medium of which the reaction is slightly alkaline, not exceeding that of the serum. If the reaction is neutral, the growth is meager, and the culture is short lived. When the reaction of a medium becomes alkaline by the addition of a small amount of sodium hydroxide, or faintly acid by the addition of a little hydrochloric acid, no growth can take place.

8. *Leptospira icterohæmorrhagiæ* is an obligatory aerobe. Any hindrance to the access of oxygen constitutes an unfavorable factor in obtaining a culture.

9. The addition of carbohydrates to media has no perceptible effect upon the growth or morphology of the organism. The reaction of the media is not modified by their presence.

10. *Leptospira icterohæmorrhagiæ* grows at any temperature between 37° and 10°C., the optimum zone being 30–37°C. Growth proceeds more rapidly at 37°C. than at 30° or at 25°, but the cultures remain viable much longer at the latter temperatures. No growth takes place at 42°C.

11. Three different media are described for the cultivation of freshly isolated strains. After prolonged cultivation on these media a strain may be readily cultivated in a serum diluted with Ringer's or isotonic salt solution.

THE SURVIVAL OF LEPTOSPIRA (SPIROCHÆTA) ICTERO-
HÆMORRHAGIÆ IN NATURE; OBSERVATIONS
CONCERNING MICROCHEMICAL REACTIONS
AND INTERMEDIARY HOSTS.

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A solution of the question of the survival of *Leptospira icterohæmorrhagiæ* in nature may be brought about (1) by following up directly the actual conditions to which the spirochetes cast off by the hosts or artificially mixed with urine or feces will have to submit, or (2) by mixing the spirochetes with each in turn of the various bacteria commonly encountered in feces, sewage, or soil, and then determining the results of their simultaneous existence in the same media. As I shall show, the spirochete of infectious jaundice is a very delicate organism and is rapidly overwhelmed by most of the bacteria from intestinal contents, sewage, or soil.

Urine in Relation to Leptospira icterohæmorrhagiæ.

The effect of urine upon the viability of *Leptospira icterohæmorrhagiæ* is of practical importance, since it has been found by previous investigators^{1, 2, 3} that the urine of about 77 per cent of the patients recovering from infectious jaundice still contains the spirochete after a period of 2 to 5 weeks. They made the interesting observation that two-thirds of the positive urines, some containing numerous spirochetes, failed to produce the infection in guinea pigs. Since the urines came from cases of 15 days' standing or longer, the fact may be ex-

¹ Ido, Y., Hoki, R., Ito, H., and Wani, H., *J. Exp. Med.*, 1917, xxvi, 341.

² Garnier, M., and Reilly, J., *Compt. rend. Soc. biol.*, 1917, lxxx, 38.

³ Cappellani, S., and Frugoni, C., *Sperimentale*, 1917, lxxi, 335.

plained by an attenuation in virulence of the organism during the course of the disease. I have seen one case in which the urine was still infective for guinea pigs 1 month after the onset of the disease.

In one series of experiments a sample of urine freshly collected from a healthy individual, who had no history of ever having had jaundice, was tested for its action upon the spirochetes. 10 cc. amounts of

TABLE I.

<i>Leptospira icterohæmorrhagiæ</i> introduced into.	Changes in appearance and reaction.	0.2 cc. of a well growing culture of American strain No. 1 inoculated into tubes containing.				
		Fluid indicated 6 cc.		Fluid indicated 3.5 cc. + rabbit serum 1.5 cc. + citrate plasma 1 cc.		
		24 hrs.	48 hrs.	24 hrs.	48 hrs.	3 days.
Normal urine.....	Clear, strongly acid.	—	—	+	+	<+
Urine 10 cc. + 0.1 N sodium hydroxide 0.1 cc.....	Clear, neutral.	<+	—	++	++	+ -
Urine 10 cc. + 0.1 N sodium hydroxide 0.2 cc.....	Precipitate +, slightly alkaline.	<+	—	++	++	+
Urine 10 cc. + 0.1 N sodium hydroxide 0.4 cc.....	Precipitate ++, moderately alkaline.	<+	—	+	+	<+
Urine 10 cc. + 0.1 N sodium hydroxide 0.8 cc.....	Precipitate +++, markedly alkaline.	—	—	—	—	—

the urine, which was strongly acid and had a titer such that 10 cc. of it required 7 cc. of 0.1 N sodium hydroxide to become moderately alkaline, were measured into a number of test-tubes, to each of which was added normal sodium hydroxide solution, the quantities added varying in each case in order to obtain a series of reactions from the original acidity of the specimen to a markedly alkaline reaction.

Cultures were set up in two parallel series, using in each series the original and partially neutralized portions of the urine, but adding to one series suitable amounts of rabbit serum and citrate plasma. Table I summarizes the results.

As the table shows, the jaundice spirochetes survived at least 24 hours in the portion of urine to which a quantity of from 0.1 to 0.4 cc. of normal sodium hydroxide solution had been added, but no trace of them could be found in the original urine or in that receiving 0.8 cc. of the alkali. In plain Ringer's solution alone the organism lived 24 hours under similar conditions. After 48 hours there were no spirochetes in any of the tubes of the first series.

The results obtained with the urine containing rabbit serum and citrate plasma were different from those of the other series. There was a good growth in all the tubes containing the unalkalized urine, and also in those to which had been added from 0.1 to 0.4 cc. of normal sodium hydroxide solution. The growth was better and lasted longer in the tubes in which the urine showed a neutral or slightly alkaline reaction than in the unmodified or more strongly alkalized urines. There was no growth in the tube to which 0.8 cc. of the normal sodium hydroxide had been added. While there was unmistakable growth in the urine media with rabbit serum and citrate plasma, the organisms were viable for only 1 week at the longest. The presence of the urine apparently reduces very much the nutrient value of the rabbit serum and citrate plasma, as is shown by the fact that the use of Ringer's solution instead of urine enables the spirochetes to multiply progressively for at least 3 weeks. Not only is the urine devoid of cultural value for the organism, but its presence in an otherwise suitable medium renders the latter less suitable for the growth of the organism.

Feces in Relation to Leptospira icterohæmorrhagiæ.

The escape in feces of living *Leptospira icterohæmorrhagiæ* from experimentally infected guinea pigs seems to be rather frequent. Ido and his coworkers,¹ for example, succeeded in producing typical spirochetosis in seven out of eleven animals tested with a corresponding number of specimens of feces; yet in spite of this high percentage of

TABLE II.

Specimens of feces from which media were prepared.	1:10 dilution.		1:10 dilution, autoclaved.		1:100 dilution, filtrate.	
	No blood.	Blood and serum added.	No blood.	Blood and serum added.	No blood.	Blood and serum added.
Normal feces No. 1	24 hrs.: A few distorted spirochetes among innumerable bacteria. No trace of the spirochetes after 48 hrs. Four daily inoculations (scarified skin) in guinea pigs all negative.	Same as preceding.	A few motile spirochetes after 24 hrs. but none after 48 hrs. No difference between normal and jaundice feces. Four daily inoculations (intrapertoneal) all positive.	Moderate multiplication of spirochetes in 4 days, which gradually increased. Four daily inoculations (intrapertoneal) all positive.	Spirochetes survived 4 days, after which they disappeared.	A good growth in 4 days which progressed well for 10 days. Three daily inoculations all positive.
" " " 2			Moderate multiplication of spirochetes in 4 days, which gradually increased. No animal inoculations.	Moderate multiplication of spirochetes in 4 days, which gradually increased. No animal inoculations.	Spirochetes survived 4 days, after which they disappeared. No animal inoculations.	A good growth in 4 days which progressed well for 10 days. No animal inoculations.
Jaundice feces No. 1			Moderate multiplication of spirochetes in 4 days, which gradually increased. Four daily inoculations. All positive.	Moderate multiplication of spirochetes in 4 days, which gradually increased. Four daily inoculations. All positive.	Spirochetes survived 4 days, after which they disappeared. One 1st day inoculation positive.	A good growth in 4 days which progressed well for 10 days. Three daily animal inoculations. All positive.

Jaundice feces No. 2			Moderate multiplication of spirochetes in 4 days, which gradually increased. No animal inoculations.	Spirochetes survived 4 days, after which they disappeared. No animal inoculations.	
					A good growth in 4 days which progressed well for 10 days. No animal inoculations.

positive results, it was by no means easy to demonstrate the presence of the spirochetes in feces with the dark-field microscope, the organism being found only once in the stools from 60 guinea pigs having spirochetosis. The specimens of feces which were infective usually contained erythrocytes. In human cases one specimen of feces out of seven was found to be infective.

There is apparently a possibility, then, that the spirochetes are excreted from patients in the feces. To determine the viability of the spirochetes under these conditions, a series of experiments was performed in which they were added in large quantities to feces, both with and without a simultaneous addition of blood ingredients. Two specimens of feces from normal individuals were used, and two from cases of jaundice in children, with the characteristic clay color. Each specimen was used in three ways: (1) as a moderately thick emulsion (1:10) in Ringer's solution, without sterilization; (2) the same sterilized by autoclave; (3) a Berkefeld V filtrate of a dilute fecal emulsion (1:100). Two sets of tubes were set up, each containing 5 cc. of one of the variously prepared suspensions. To each of the tubes of one set were added a few drops of defibrinated blood and 1 cc. of serum from a normal rabbit, and to all the tubes were added 2 cc. of a richly growing culture of either the American or the European strain of the organism. Both sets of tubes were then placed at a temperature of 26°C.

The fate of the spirochetes under these conditions was followed daily by direct microscopic examination and indirectly by inoculation tests on guinea pigs. Table II summarizes the results obtained with the European strain. The results with the American strain were practically identical and are consequently not recorded here.

As is apparent from the table, the spirochete cannot survive in a fecal emulsion, even when there are present sufficient nutrient elements, longer than 24 hours at a temperature of 26°C. That this fact is due to the simultaneous presence of various bacteria, which rapidly overgrow the delicate spirochetes and deprive them of the necessary nutrient substances, is inferred from the much longer survival of the spirochetes in the tubes containing the sterilized emulsion, particularly in those to which were added the blood and serum. In the latter tubes, in fact, there was a temporary multiplication of the

organisms lasting several days. In the tubes containing a dilute, sterile, fecal filtrate, the spirochetes survived at least 4 days, and the addition of blood and serum caused the filtrate to become a suitable culture medium; if not equally as good as Ringer's or saline solution. Inoculations made with the mixtures of the non-sterilized fecal emulsions, with and without the blood and serum and the spirochetes, applied to the scarified skin of guinea pigs, were all negative. Where there was an actual multiplication or survival of the organism, as in the case of sterile suspensions or filtrates, with or without the addition of blood, the animal inoculations were positive.

Judging from the foregoing experiments, it seems highly improbable that, under natural conditions, the causative agent of ictero-hemorrhagic spirochetosis survives for any length of time after it has left the human body in the feces. It is probably rapidly destroyed by the common bacterial flora of the intestinal tract.

Polluted Water and Soil in Relation to Leptospira icterohæmorrhagiæ.

Samples of water were collected from the East River (a tidal river), from sewage, and from a stagnant cesspool in New York City. It is needless to say that such water is highly contaminated with various bacteria. In one series of experiments the water was used as it was, in another it was autoclaved in order to destroy contaminating bacteria, and in another it was filtered. An emulsion of freshly excreted horse stool was used in one series. The experimental data are given in Table III.

The results show that the spirochetes are not capable of multiplying or even of surviving for any length of time in these contaminated waters. They invariably disappeared in 48 hours. Even when the contaminating bacteria were removed by autoclaving or filtration and rabbit serum was added, only indifferent media resulted, and without the addition of an adequate amount of a suitable nutrient medium (rabbit serum in this experiment) no culture could be obtained.

The question of how long a rich culture of the spirochetes will remain viable when mixed with distilled water and left unprotected from dust in a room was next determined. A Flanders strain, having grown luxuriantly in rabbit serum, Ringer's solution, and agar mix-

TABLE III.

<i>Leptospira icterohæmorrhagiæ</i> introduced into.	Growth of bacteria.	Growth of spirochetes.	Survival of spirochetes.
Ringer's solution 4.5 cc. + rabbit serum 1.5 cc. (control)..	—	+++	Many wks.
East River water 6 cc.....	+++	—	
“ “ “ 4.5 cc. + rabbit serum 1.5 cc.....	+++	—	
“ “ “ autoclaved, 6 cc.....	—	—	
“ “ “ “ 4.5 cc. + rabbit serum 1.5 cc.....	—	+	2 wks.
Sewer water 6 cc.....	+++	—	
“ “ 4.5 cc. + rabbit serum 1.5 cc.....	+++	—	
“ “ autoclaved, 6 cc.....	—	—	
“ “ “ 4.5 cc. + rabbit serum 1.5 cc...	—	+++	Many wks.
Stagnant water 6 cc.....	+++	—	
“ “ 4.5 cc. + rabbit serum 1.5 cc	+++	—	
“ “ autoclaved, 6 cc.....	—	—	
“ “ “ 4.5 cc. + rabbit serum 1.5 cc.	—	+++	Many wks.
Horse stool emulsion 6 cc.....	+++	—	
“ “ “ 4.5 cc. + rabbit serum 1.5 cc.....	+++	—	
“ “ “ autoclaved, 6 cc.....	—	—	
“ “ “ “ 4.5 cc. + rabbit serum 1.5 cc.....	—	+(?)	Accidentally contaminated.
Sewer filtrate 6 cc.....	—	—	
“ “ 4.5 cc. + rabbit serum 1.5 cc.....	—	++	More than 3 wks.

ture for 22 days, was placed in distilled water (ten times the volume of the culture) and then allowed to stand in the laboratory without being covered. The distilled water was not sterile, but contained a few large motile bacilli. The results were as follows:

24 hrs: Spirochetes +++; active and long; numerous motile bacilli; fluid slightly opalescent
 48 hrs.: Spirochetes +++; active; more bacilli.
 3 days: “ ++; “ “ “
 4 “ “ +; “ probably more bacilli.
 5 “ “ +; many immobile; “ “
 6 “ “ <+; nearly all dead.
 7 “ “ —.

The spirochetes remained active and numerous for 48 hours, but all of them gradually disappeared within a week. A drinking water, therefore, richly contaminated with spirochetes, will not be infectious longer than a week.

Samples of soil were collected from several localities in and about New York City for use in an experiment performed to ascertain how long soil will harbor spirochetes under experimental conditions. The samples were rich in organic matter and some came directly from fertilized ground. They were all neutral in reaction. One specimen of soil was obtained from a deeper stratum than the others and was yellowish gray in color. All were purposely contaminated with the spirochetes and determinations of their continued presence in it made daily. No spirochetes could be detected after 72 hours, while there was always an abundance of bacteria. The spirochetes seem to be rapidly overgrown by the contaminating bacteria.

Various Bacteria in Relation to Leptospira icterohæmorrhagiæ.

When the spirochetes are excreted from the infected host, either in the feces or in the urine, their immediate fate will depend upon the presence of various putrefactive bacteria which are always found in the soil in which the feces or urine is deposited. Today we know all of the more common varieties of bacteria that inhabit the intestinal tract or that may be found in unclean objects or soil. There are, of course, a great number of anaerobes as well as aerobes, but since the spirochete in question is an obligatory aerobe,⁴ the study of the relation of the bacteria to it becomes much simpler. We have, therefore, to direct our attention only to the part played by aerobic bacteria under natural conditions.

There are many ways of conducting such a study, but I have chosen an indirect one; namely, that of observing the effect of the simultaneous presence of the spirochete in question and each in turn of those bacteria which are likely to coexist with it at the moment when the infected feces, urine, or dead rodent becomes subject to the decomposing forces of the organic world.

A number of culture tubes containing media suitable for the growth

⁴ Noguchi, H., *J. Exp. Med.*, 1918, xxvii, 593.

of spirochetes was prepared, and all were inoculated with the organism. The tubes were then inoculated with various bacteria and placed in a

TABLE IV.

Bacteria.	Growth of bacteria.	Growth of spirochetes.	Survival of spirochetes.	Remarks as to hemolysis in media.
Control without bacteria.....	—	+++	Many wks.	—
<i>B. fæcalis alkaligenes</i>	+	+++	12 days.	—
<i>B. aerogenes</i>	++	—	48 hrs.	+
<i>B. cloacæ</i>	++	—	24 "	<+
<i>B. coli</i>	++	—	24 "	<+
<i>B. dysenteriae</i> Shiga.....	+	<+	48 "	—
<i>B. " Flexner-Harris</i>	<+	+	48 "	—
<i>B. typhosus</i>	<+	<+	48 "	—
<i>B. paratyphosus</i> A.....	++	—	24 "	—
<i>B. " B</i>	++	—	24 "	—
<i>B. prodigiosus</i>	++	—	24 "	+
<i>B. proteus vulgaris</i>	++	—	24 "	+
<i>B. pyocyaneus</i>	++	—	24 "	+
<i>B. suispestifer</i>	++	—	24 "	—
<i>B. suicidus</i>	++	—	24 "	—
<i>B. subtilis</i>	++	—	24 "	<+
<i>B. mesentericus</i>	+	—	24 "	<+
<i>B. xerosis</i>	+	—	24 "	—
<i>B. sp.? large, motile, chromogenous</i>	+	++	4 days.	—
<i>Streptococcus</i> Pr.....	<+	++	5 "	—
" Brown F 17.....	++	—	24 hrs.	—
" " A 1.....	=	++	5 days.	—
" " C 2.....	+	++	8 "	—
" " W 18.....	++	<+	3 "	—
" " K 4.....	++	++	5 "	+
" " S 6.....	++	<+	6 "	+
" " H 6.....	+	—	24 hrs.	+
<i>Pneumococcus</i> Type I.....	++	<+	3 days.	—
" " II.....	++	—	24 hrs.	—
" " III.....	+	<+	3 days.	—
" " IV.....	+	—	24 hrs.	—
<i>Streptococcus aureus</i>	++	+	48 "	<+ slowly.
" <i>albus</i>	++	+	48 "	—

thermostat at the temperature of 26°C. The culture media consisted of 1.5 cc. of rabbit serum, 4.5 cc. of Ringer's solution, 1 cc. of citrate plasma, and 1 drop of defibrinated rabbit blood. Observations were

made of the growth, survival, or disappearance of the spirochetes, the growth of the bacteria, and the presence or absence of hemolysis in the cultures. The results obtained during a period of 2 weeks are recorded in Table IV.

It is apparent from the recorded observations that the more vigorous the growth of a bacterium, the less is the possibility that the spirochetes in the same medium will multiply. The longest period of survival of the spirochetes, except in the control tubes, was observed in the media simultaneously inoculated with *Bacillus fæcalis alkaligenes*. Certain strains of streptococci, notably the non-hemolytic types, seem not to have interfered for a certain period, after which, however, the spirochetes rapidly disappeared from the culture. In the presence of most of the intestinal bacteria, such as *Bacillus coli*, *Bacillus aerogenes*, *Bacillus cloacæ*, etc., the spirochetes were not only unable to multiply but were rapidly destroyed within 24 hours. It may be added that no growth of the spirochetes took place in ordinary bouillon, either with or without the simultaneous inoculation of the bacteria just enumerated. The bacteria grew vigorously in the bouillon.

Microchemical Reactions.

The resistance of various spiral organisms to the solvent action of bile, bile salts, saponin, and sodium oleate has been a subject of study for many years, and it was once thought to differentiate the protozoa from the bacteria. Although this view is no longer valid, because some bacteria have been found to act like protozoa and *vice versa*, the fact is of sufficient interest to make worth while a determination of the resistance of the present organism to these reagents (Table V).

The jaundice spirochetes appear to be highly sensitive to the destructive action of the bile⁵ and bile salts when employed in concentrations of 1:30 or more, while saponin exhibited no injurious effect upon them, even when used in as high a concentration as 10 per cent. The action of sodium oleate was stronger than that of the bile or bile salts and produced a granular disintegration of the organism in a dilution of 1:10,000. Among the organisms which under-

⁵ Garnier, M., and Reilly, J., *Compt. rend. Soc. biol.*, 1917, lxxx, 41.

TABLE V.

Reagent.	Results in different concentrations.									
	1:10		1:30		1:100			1:300		1:1,000
	After 5 min.	After 30 min.	After 5 hrs.	After 5 min.	After 5 min.	After 30 min.	After 5 hrs.	After 5 hrs.	After 5 hrs.	After 5 hrs.
Ox bile.	Still active.	None motile, nearly all shadow forms.			All shadow forms.		Some affected. Nearly all active.	No effect. All active.		
Rabbit bile.	Many inactive.	All shadow forms.	All shadow forms.		All shadow forms.		All shadow forms.	Some affected. Majority active.		
Sodium taurocholate.	All shadow forms.	Shadow forms.	Shadow forms less distinct.	All immobile. Better preserved in form.	Nearly all active.	All shadow forms.		Nearly all active.	No effect. All active.	
Sodium glycocholate.	Nearly all shadow forms.		All shadow forms.				Some still active.	Nearly all active.	No effect. All active.	
Sodium oleate.	All dead; distorted and granular.			All dead; distorted and granular.			Nearly all gone; few motile.	Nearly all gone; few motile.	Nearly all gone; few motile, but more active than those in the 1:300 dilution.	
Saponin.			No effect. All active.		No effect. All active.		No effect. All active.	No effect. All active.		

went this disintegration, however, was a number of actively motile, apparently intact organisms.

The destructive action of the rabbit bile as well as of the bile salts and sodium oleate was considerably reduced by the addition of serum, as shown in Table VI.

Ido and his coworkers¹ observed that in spite of the difficulty of finding spirochetes in the bile when it was examined under the dark-field microscope, two out of three specimens of the bile of guinea pigs dying of experimental spirochætosis icterohæmorrhagica were capable of producing typical infection in the guinea pig. This

TABLE VI.

<i>Leptospira icterohæmorrhagica</i> introduced into.	10 per cent rabbit bile 1 cc. + culture 1 cc.	10 per cent sodium taurocholate 1 cc. + culture 1 cc.	10 per cent sodium oleate 1 cc. + culture 1 cc.	0 + culture 1 cc.
Rabbit serum 0.5 cc.	No apparent ef- fect. All ac- tive.	Nearly all ac- tive.	Nearly all ac- tive.	All motile.
60 per cent rabbit serum 0.5 cc.	Many gone, some motile.	Many dead and distorted. A few motile.	Many active.	" "
20 per cent rabbit serum 0.5 cc.	Nearly all gone.	Nearly all gone.	" "	" "
6 per cent rabbit serum 0.5 cc.	All gone.	All gone.	Nearly all gone.	" "
2 per cent rabbit serum 0.5 cc.	" "	" "	All gone.	" "
Ringer's solution 0.5 cc.	" "	" "	" "	" "

may be ascribed to the fact that in these specimens of bile there was mixed a certain amount of the blood and also the serous exudate from the affected liver, which, by virtue of their well known inhibitory effect upon the solvent action of the bile salts, must have protected some spirochetes from destruction in the bile. Guinea pig bile was affected by the serum in the same way.

A parallel series of experiments with a specimen of ox bile obtained from an abattoir gave somewhat contradictory results. In this instance the addition of the rabbit or horse serum failed to check the destruction of the organism by this bile, which had a much stronger

solvent power than that of the rabbit or guinea pig. At all events, the amount of the serum necessary to nullify the destructive action of the bile is so large that the escape of the spirochetes in the bile seems less probable than would appear from the observations of the investigators just quoted. Perhaps the impairment of hepatic function through the spirochetal infection of the organ may lead to a decrease of the bile salts in such a specimen.

Leptospira icterohæmorrhagiæ and Intermediary Hosts.

It has been shown by previous investigators that the spirochetes may remain in the organs of certain rodents without producing serious illness, and that they may be excreted in the urine. From the experiments already described, it seems improbable that the spirochete can survive very long after leaving the infected hosts. The infection of man, therefore, must result from contact with the spirochete before its destruction under natural conditions; that is, the carrier rodents must be present in places frequented by man. But while this source of infection may explain many cases of infection, there are a few in which the infective agent cannot be traced in this way.

The question of insect carriers has been taken up by Reiter,⁶ who obtained only negative results with certain biting flies, fleas, and bed-bugs. In the present study opportunities were afforded the writer to ascertain whether or not the larvæ of certain varieties of flies or mosquitoes could become infected with spirochetes when fed on infected guinea pig liver or raised in a stagnant water tank into which an abundance of the culture had been put.

The larvæ of the common house-fly were allowed to feed for 2 days on infected material consisting of several pieces of the liver and kidney of a guinea pig killed in the last stage of experimental Weil's disease. They were then transferred to a clean receptacle and fed for 5 days on a non-infected mass of horse manure, and at the end of that time they were crushed into an emulsion and smeared over depilated areas of the skin of guinea pigs. The emulsion was also examined for spirochetes under the dark-field microscope. The examination revealed no spirochetes, and the guinea pig remained normal.

⁶ Reiter, H., *Deutsch. med. Woch.*, 1916, xlii, 1282.

A similar experiment with the larvæ of bluebottle flies (*Calliphora vomitoria*) gave only negative results.

In another series of experiments, about 50 cc. of a rich culture of spirochetes (Japanese strain) were added to 150 cc. of stagnant water in which twenty-five mosquito larvæ had been living for some time. The water was neutral in reaction and was quite clear and transparent at the time when the culture was introduced. The larvæ swam about actively in the usual manner after the addition of the culture. A drop of the contaminated water examined under the dark-field microscope contained numerous active spirochetes. There were a few bacteria. After 24 hours at room temperature, the water became somewhat turbid. Most of the larvæ were still active, but the number of the spirochetes was diminished and that of the bacteria increased. At the end of 48 hours there was a scum of bacteria over the surface of the water and no spirochetes could be found. All but six of the largest larvæ had died. The water was full of bacteria and infusoria. It is possible that the death of the mosquito larvæ and of the spirochetes was the result of overcrowding by the bacteria and infusoria, increased suddenly by the addition of the culture media to the water. The surviving larvæ were kept in the same water for 5 days and then crushed into an emulsion to be used for an infection experiment on a guinea pig and also for examination under the dark-field microscope. The results were entirely negative.

Another series of experiments was performed with adult mosquitoes (*Culex pipiens*) by first allowing them to feed on an infected guinea pig, in the blood of which had been found spirochetes, and then, after 6 days, causing them to bite normal guinea pigs. No infection resulted from their bites.

Wood ticks (*Dermacentor andersoni*) failed in several experiments to transmit the infection from guinea pig to guinea pig. Leeches (*Hirudo medicinalis*) were allowed to suck blood from an infected guinea pig until their bodies were engorged. In the blood escaping from the wound inflicted by the leeches a few spirochetes could be found under the dark-field microscope. These "infected" leeches were kept at room temperature for 7 days and afterwards in a cool room at 15°C., being taken out at the end of intervals of 2, 3, 4, 6, and 8 months and made to suck normal guinea pigs, but so far no infection

has been produced. Some of the leeches died in the meantime, but those which still survived at the end of the 3 month interval were examined for the presence of spirochetes. The viscid, dark reddish, decomposed (?) blood showed no spirochetes under the dark-field microscope, nor did it cause infection when tested on guinea pigs. Some of the tissues were examined by the silver impregnation method, but with negative results. Apparently there is no multiplication of the spirochetes after their ingestion by leeches, and no infection can be induced by the bite of the latter after a period of 1 week.

SUMMARY AND CONCLUSIONS.

1. *Leptospira icterohæmorrhagiæ* is unable to grow in the urine, either with or without the addition of suitable culture ingredients, the acidity of the urine being detrimental to the growth. It survives less than 24 hours, unless the urine is neutralized or slightly alkalized, when the period of survival is somewhat longer. If suitable nutrient ingredients are added to the neutralized or slightly alkalized urine, the organism is able to grow for about 10 days, after which multiplication ceases.

2. Feces from normal or jaundiced persons destroy *Leptospira icterohæmorrhagiæ* within 24 hours when a rich culture is added and the mixture allowed to stand at 26°C. The addition of blood serum and corpuscles does not prevent the destruction of the organism. Autoclaved specimens and filtrates of unheated feces do not constitute a suitable medium in which to keep the organism alive for any length of time, but the addition of blood corpuscles and serum in adequate quantities renders them fairly satisfactory as media. Under natural conditions *Leptospira icterohæmorrhagiæ* cast off in the feces cannot survive more than 24 hours.

3. Polluted water, sewage, and soil will not serve to keep *Leptospira icterohæmorrhagiæ* alive for more than 3 days at the most. When deprived by filtration or autoclaving of their bacteria they become indifferent diluents and may be used to make up a culture medium when mixed with serum and citrate plasma of a suitable animal. Sterilized soil with a neutral reaction, when added to a culture, has an unfavorable effect upon the growth of the organism.

4. Most of the aerobic bacteria found in feces, sewage, soil, and tap water inhibit the growth of *Leptospira icterohæmorrhagiæ* when inoculated into the same medium. *Bacillus fæcalis alkaligenes* and many strains of non-hemolytic streptococci caused the least interference, although growth was never so vigorous or lasting in the media in which they were present as in the control media. Certain pathogenic bacteria (*Bacillus typhosus*, *Bacillus paratyphosus*, *Bacillus dysenteriæ*, pneumococcus) are antagonistic to the growth of the spirochete.

5. *Leptospira icterohæmorrhagiæ* is highly sensitive to the destructive action of bile, bile salts, and sodium oleate, but resists the action of saponin. In this last respect it differs from many so called spirochetes. The destructive action of these agents is counteracted by blood serum.

6. The larvæ and adults of the *Culex* mosquito, the larvæ of the house-fly and bluebottle fly, wood ticks (*Dermacentor andersoni*), and leeches failed to become carriers of the spirochetes when fed on infected guinea pigs or their organs; that is, they cannot play the part of an intermediary host of *Leptospira icterohæmorrhagiæ*.

BEHAVIOR OF HYPOCHLORITE AND OF CHLORAMINE-T SOLUTIONS IN CONTACT WITH NECROTIC AND NORMAL TISSUES IN VIVO.

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It has been known for some time that the chlorine content and consequently the potency of hypochlorite of soda solutions diminishes rapidly when in contact with the surface of wounds. This is emphasized by Carrel and Dehelly, and for this reason they advocate a frequent renewal of the antiseptic solution to the wound.¹ This insures that the concentration shall be kept as constant as possible.

It would be difficult to determine the rapidity of the fall in chlorine concentration on an actual wound as encountered in the ward, and almost impossible to parallel such observations with others on an equal quantity of solution in contact with an equal area of normal skin. Inasmuch as exact determinations of the rapidity of the fall in chlorine concentration on pathological and on normal skin, under experimental conditions, might be of value to surgeons using Dakin's hypochlorite and chloramine-T solutions clinically, we chose the following method of investigation.

The left ears of three white rabbits of the same relative size and weight were exposed to the rays emitted by a Coolidge tube. The spark-gap used measured 3 inches; the milliamperage was 10; the distance from the target to the ear was 6 inches; and the time of exposure was 20 minutes.

8 weeks later the x-rayed ears each exhibited a sharply demarcated gangrenous area over which there were considerable crusting of epithelium and secretions and in the lumen there was much thick pus.

The ears of the affected rabbits were each suspended for 20 minutes in a beaker containing 400 cc. of the solution to be tested.

¹ Carrel, A., and Dehelly, G., *The treatment of infected wounds*, New York, 1917, 65.

Rabbit 1.—Right and left ears suspended in separate beakers containing Dakin's hypochlorite solution, made from bleaching powder, 10 cc. of which required 13 cc. of 0.1 N sodium thiosulfate solution for reduction (0.48 per cent sodium hypochlorite).

Rabbit 2.—Right and left ears suspended separately in beakers containing a solution comparable in alkalinity with properly made Dakin's hypochlorite solution—sodium carbonate 1 gm. and sodium bicarbonate 17 gm. per liter of water.

Rabbit 3.—Right and left ears suspended separately in chloramine-T² solution which required 12.75 cc. of sodium thiosulfate for reduction (about 2 per cent chloramine-T), and contained approximately the same proportion of available chlorine as the hypochlorite solution used on Rabbit 1.

TABLE I.

Solution.	In contact with.	Before.	Immediately after.	2 hrs. after	17 hrs. after.
		cc.	cc.	cc.	cc.
Dakin's hypochlorite solution.....	Normal ear.	13.00	12.35	12.15	11.50
“ “ “	Gangrenous ear.	13.00	11.55	10.30	8.65
“ “ “	Control (no tissue).	13.00	13.00	13.05	12.60
Carbonate-bicarbonate “	Normal ear.				
“ “ “	Gangrenous ear.				
Chloramine-T solution.....	Normal ear.	12.75	12.75		12.75
“ “	Gangrenous ear.	12.75	12.75		12.35
“ “	Control (no tissue).	12.75	12.75		12.75

In the tables the figures represent the number of cubic centimeters of 0.1 N sodium thiosulfate solution required to reduce the chlorine in 10 cc. of the solution.

Table I shows the titration figures before, immediately after the 20 minutes' exposure of the normal and necrotic ears to the solutions, 2 hours after the ears had been removed from the solutions, and 17 hours after removal. The solutions were kept in covered vessels at room temperature in the interval between titrations.

The fall in chlorine concentration was more rapid in the Dakin's hypochlorite solution applied to the gangrenous ear than in that applied to the normal ear. The fall in concentration, however, was not complete immediately after the ears were removed from the solution but became more pronounced the longer the interval between

² Prepared by the Abbott Laboratories, Chicago.

the removal of the ears and the titration. The titration of the control solution, which had not been exposed to any tissue, demonstrated a fall in the titration figure from 13 to 12.60 cc., and a small proportion of the loss in chlorine of the solutions in contact with the tissues might be explained by this spontaneous deterioration of the unstable hypochlorite solutions. However, this factor is insufficient to account for the fall from 13 cc. before exposure to 8.65 cc. 17 hours later in the gangrenous ear, nor from the same figure before to 11.50 cc. at the end of the 17 hour interval in the normal ear. The fall from 13 cc. before exposure to 11.55 cc. immediately afterward is associated with the erosive action of the hypochlorite solutions, which we have measured quantitatively in a former investigation,³ but the cause of the further fall to 8.65 cc. is not immediately clear. The fluid in contact with the gangrenous ear was cloudy immediately after the removal of the ear. This cloudiness of the fluid was not so marked at the end of the 2 hour interval when the second titration was made, and the fluid was almost as clear as the control at the end of the 17 hour interval when the last titration gave the lowest chlorine concentration recorded. Close inspection of the fluid immediately after the removal of the ear revealed the presence of small particles of necrotic tissue, flecks of pus, etc., in suspension. These became less noticeable the longer the antiseptic solution was allowed to act. The fall in chlorine concentration exhibited immediately after the removal of the ears was due to the erosive effect of the solution on the necrotic tissue, and to its combination with the products of the tissues *in situ*. However, during this action, appreciable particles of necrotic tissue, agglomerations of pus cells, and little gummy concretions made up of dried serum, epithelial cells, etc., were separated from the necrotic ear and it is the subsequent reaction of the hypochlorite with these which caused the continued fall in the chlorine titer. That the chlorine is directly concerned in this solvent action seems assured from former experiments reported.³ In the course of the reaction the chlorine probably goes into such stable union with the protein substances that it is not available to the sodium thiosul-

³ Taylor, H. D., and Austin, J. H., The solvent action of antiseptics on necrotic tissue, *J. Exp. Med.*, 1918, xxvii, 155.

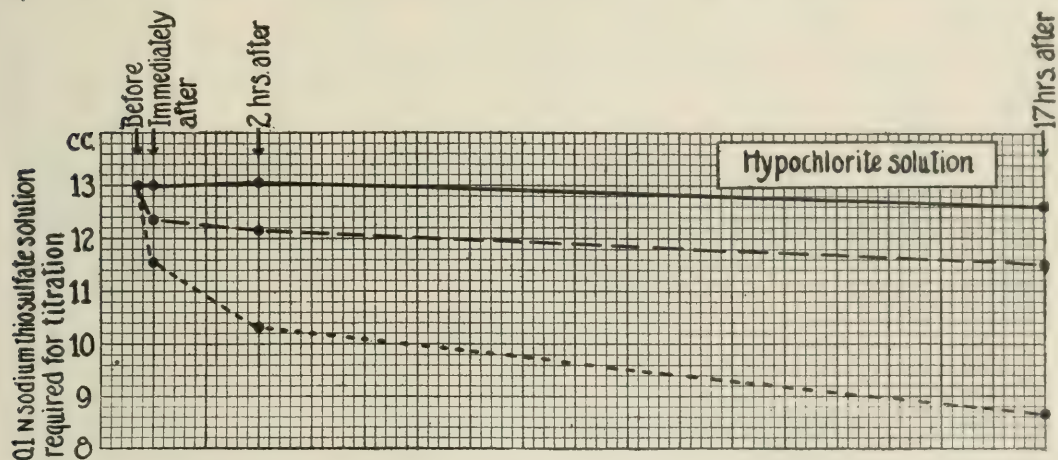
fate solution, and thus the fall in titer measures the exact quantity of chlorine used up in the reaction with the wound products.

The fall in chlorine concentration of the hypochlorite solution in contact with the normal ear, much less pronounced than with the x-rayed ear, was probably due to the erosive action of the solution on the hair and superficial epithelium of the normal ear and the slow digestion of the particles removed caused the slow fall noted over the interval of 17 hours. As there was much less tissue capable of reacting with the solution in the normal than in the gangrenous ear, the titration figures were higher at all observations in the solution exposed to the former. Close observation revealed the erosive effect on the ear itself.

The fall in chlorine concentration noted in the chloramine-T solutions was much less than that observed in the hypochlorite solutions. This corresponds with the greater stability of the former and with their lack of erosive effect on necrotic tissue.³ It is interesting to note that there was no fall in chlorine concentration in the chloramine-T solution applied to the normal ear, and correspondingly no erosive action on the hair or superficial epithelium was demonstrable. The solution was likewise clear when removed from the ear and throughout the period of observation, in contrast to the hypochlorite solution which was at first cloudy and only late in the experiment became relatively clear. The titration figures after 17 hours were approximately the same as those made immediately after the removal of the ears from the solution. Text-fig. 1 shows graphically the fall in chlorine concentration in the hypochlorite solution applied to the gangrenous ear, in that applied to the normal ear, and in the control solution which was not allowed to act on any tissue. Text-fig. 2 gives comparable curves for the chloramine-T solutions.

A weaker hypochlorite solution, titrating 9 cc. of sodium thiosulfate (0.1 N), was applied to the gangrenous ear and to the normal ear of Rabbit 1, and titration figures before, immediately after removal of the ears from the solution, 2 hours afterward, and 17 hours afterward were compared with a control solution which was never in contact with tissue. These results, shown in Table II and Text-fig. 3, confirm those shown in Table I and Text-fig. 1 for Rabbit 1.

The ears of each rabbit were then suspended in solutions of the same types and concentrations as those shown in Table I for 7 consecutive days, the period of exposure on each day being 20 minutes. At the end of this time it was seen that the gangrenous ear suspended in Dakin's hypochlorite solution had cleared up proportionately more



———— Control; no tissue.

----- Normal ear.

..... X-rayed ear.

TEXT-FIG. 1. The fall in chlorine concentration in the hypochlorite solution applied to the gangrenous ear, in that applied to the normal ear, and in the control solution which was not allowed to act on any tissue.

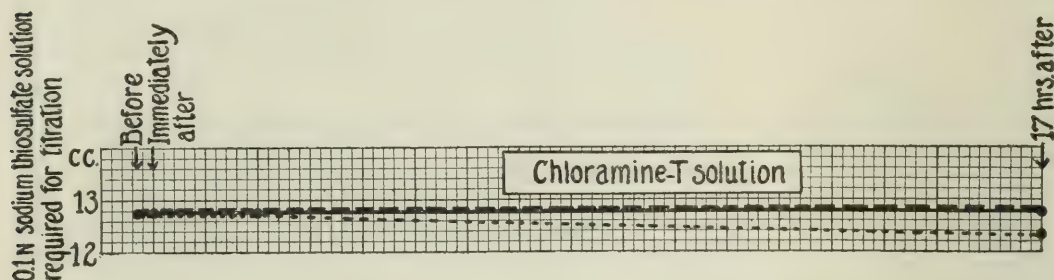
TABLE II.

Solution.	In contact with.	Before.	Immediately after.	17 hrs. after.
		cc.	cc.	cc.
Dakin's hypochlorite solution.....	Normal ear.	9.0	8.5	8.25
" " ".....	Gangrenous ear.	9.0	7.0	5.5
" " ".....	Control (no tissue).	9.0	9.0	8.9

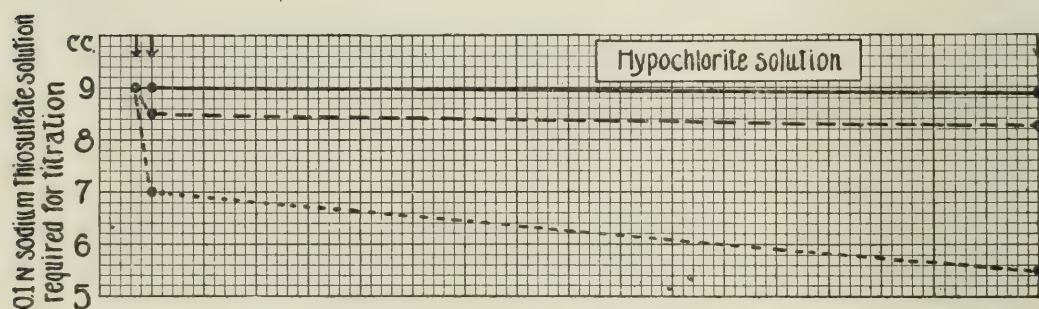
than the gangrenous ears of either of the other rabbits. The scabs were more eroded and the pus present was less in amount than in either of the others. The gangrenous area looked much cleaner. The chloramine-T gangrenous ear, which was not so severe an x-ray burn in the beginning, had not improved appreciably. The gangrenous ear

treated with the control alkaline solution had not changed in appearance.

The normal ears of the chloramine-T and of the alkaline control rabbits were just as they had been before treatment with these substances. The normal ear treated with the hypochlorite solution was



TEXT-FIG. 2.



TEXT-FIG. 3.

TEXT-FIG. 2. The fall in chlorine concentration in the chloramine-T solution applied to the gangrenous ear, in that applied to the normal ear, and in the control solution which was not allowed to act on any tissue.

TEXT-FIG. 3. The fall in chlorine concentration of the second hypochlorite solution, titrating 9 cc. of sodium thiosulfate (0.1 N), applied to the gangrenous ear, in that applied to the normal ear, and in the control which was not allowed to act on any tissue.

intensely inflamed. It was twice as thick as it had been before it was treated, due to intense edema. Congestion was marked and the surface temperature was higher than normal. There was superficial ulceration in places and petechiæ were scattered through the subcutaneous tissues.

CONCLUSIONS.

1. The fall in chlorine concentration of Dakin's hypochlorite solution is more rapid in contact with necrotic than in contact with normal tissue.

2. The fall in chlorine concentration of chloramine-T solution is very slight when applied to necrotic tissue and is negligible when applied to normal tissue.

3. The action of the hypochlorite solution on tissue results in the separation of particles of necrotic tissue, hair, epithelial scales, coagulated serum, etc., and a gradual digestion of these substances, taking place over a period of at least 17 hours.

4. The fall in the chlorine concentration of the hypochlorite solution is not complete until the particles are completely dissolved.

5. Chloramine-T solution, 2 per cent, has no erosive effect comparable with that exhibited by the hypochlorite solution.

6. Repeated exposures to the three solutions show the hypochlorite solution to be superior in its cleansing ability on necrotic tissue.

7. The hypochlorite solution is much more irritating to normal rabbit skin than chloramine-T solution or the alkaline control solution.

8. Therefore, the irritating effects must be due to the readily available chlorine.

TOXICITY OF CERTAIN WIDELY USED ANTISEPTICS.

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(Received for publication, March 23, 1918.)

In view of the widespread use of certain antiseptics in the treatment of infected wounds, it has seemed desirable to make toxicity tests on animals under conditions in which rapid absorption might be expected. While, as a rule, the antiseptics are employed under conditions that preclude the possibility of much absorption with consequent systemic effect, yet occasionally certain of them have been recommended and in some instances even used for injection into closed cavities. This practice, which would probably lead to considerable absorption, has not been general, the majority of surgeons proceeding with much caution.

A few experiments of Carrel and Dehelly¹ demonstrated that Dakin's hypochlorite solution when injected subcutaneously in the guinea pig was relatively non-toxic, one-fortieth of the body weight of the animal injected being borne without demonstrable ill effect. Bashford² has tested the toxic effect of dilute hypochlorite solutions on the living tadpole immersed in Dakin's solution. Inasmuch as these experiments were few and no data for comparing the relative toxicity of a series of antiseptics were given, it was decided to investigate the toxic action of a number of antiseptic substances in common use. The method was to inject increasing doses into mice intraperitoneally and into guinea pigs both subcutaneously and intraperitoneally, and to note and tabulate the results.

Method.

White mice of approximately 20 gm. and guinea pigs of 300 to 600 gm. body weight were employed and the amount of chemical used is

¹ Carrel, A., and Dehelly, G., *The treatment of infected wounds*, New York, 1917, 32.

² Bashford, E. F., *Lancet*, 1917, ii, 595.

based on the weight of each animal. In regard to the chlorinated antiseptics, the lethal dose is calculated in terms of the sodium hypochlorite equivalent and the available chlorine of the antiseptic. The percentage of the total weight of the antiseptic substance represented by the sodium hypochlorite equivalent is given in a foot-note to Table I.

In Table I the results of the intraperitoneal injection in mice of increasing doses of the antiseptics studied are tabulated. In Column A of Table IV will be found a condensed summary of these results, the antiseptics being arranged in the order of their decreasing toxicity for the animals. Control injections of four of the vehicles employed, namely water, isotonic saline solution, sterile paraffin oil, and Dakin and Dunham's bland oil solvent for dichloramine-T (chlorcosane³), show these to be well borne in larger doses than those employed in any of the injections with antiseptics into the test animals. Eucalyptol, however, which has been used in combination with paraffin oil as a vehicle for dichloramine-T is so toxic that its use in experiments of the nature of those recorded here is impossible. The diluting vehicle has been water or isotonic saline solution with all antiseptics except dichloramine-T.

In Table I are given in detail the results of the experiments in which mice were used. The nature and strength of the solution injected, the amount of the solution in cubic centimeters, the amount of the drug in actual milligrams administered and in milligrams per 100 gm. of body weight, with the final results, are recorded.

Table II gives in the same way the results obtained with the few guinea pigs that were injected subcutaneously.

In Table III are recorded the results of the experiments in which guinea pigs were injected intraperitoneally. The form of the table is the same as in Tables I and II.

Table IV summarizes the results given in Tables I to III, showing the greatest dose per 100 gm. of body weight that the animals were able to survive and the smallest dose necessary to kill with the antiseptics named.

³ The chlorcosane was kindly given us by Dr. H. D. Dakin and Dr. E. K. Dunham. Dakin, H. D., and Dunham, E. K., *Brit. Med. J.*, 1918, i, 51.

DISCUSSION.

It will be seen from the tables that the only antiseptic of which the smallest fatal dose was smaller than the largest survival dose was dichloramine-T. Since two mice survived 4.7 mg. per 100 gm. of body weight, it is probable that 15.5 mg. rather than 1.6 mg. is to be considered the smallest fatal dose for this series. The distribution of the drug in the viscid bland oil used as a vehicle is probably uneven, which may account for the somewhat variable results obtained with this antiseptic both in mice and in guinea pigs.

Of all the substances tested, eucalyptol and brilliant green are the most toxic, the lethal dose of each being 0.1 mg. per 100 gm. of body weight. Mercurophen,⁴ mercuric chloride, and chloramine-T constitute the group with the next highest toxicity, the lethal dose being 1 mg. per 100 gm. of body weight. Dichloramine-T, proflavine,⁵ and the four hypochlorite solutions tested follow in the order named with a lethal dose of about 10 to 15 mg. per 100 gm. of body weight. The least toxic chemicals are iodine and phenol, of which the lethal doses are about 50 mg. per 100 gm. of body weight.

In Table II are recorded a few experiments with the antiseptics injected under the skin of the abdomen of guinea pigs. The lethal dose of Dakin's hypochlorite solution per 100 gm. of body weight is the same as that determined intraperitoneally in the mouse. Chloramine-T and dichloramine-T administered in this manner gave rise to local necrosis with extensive sloughing. It is probable that only a small part of the drug injected reached the general system of the animal and in consequence the determination of the lethal dose in this way can hardly be considered satisfactory. It was accordingly abandoned and five of the antiseptics were tested in guinea pigs by intraperitoneal injections. The results are tabulated in Table III and summarized in Column B of Table IV.

Chloramine-T has the same toxicity per unit of body weight for guinea pigs and for mice. The same may be true of dichloramine-T or this substance may be somewhat less toxic for the guinea pig.

⁴ The mercurophen was sent to us for trial through the kindness of Dr. J. F. Schamberg.

⁵ The proflavine was obtained from England.

TABLE I.
Results of Injecting Mice Intraperitoneally.

Animal No.	Solution			Amount of solution.	Measured in terms of.		Amount of drug.	Amount of drug per 100 gm. of body weight.	Result.
				cc.			mg.	mg.	
1	Sodium chloride 0.85 per cent.			0.50	Sodium chloride.		4.20	20.00	Lived.
2	" " 0.85 "			1.00	" "		8.50	40.00	"
3	" " 0.85 "			4.00	" "		34.00	170.00	"
4	Distilled water.			0.50					Lived.
5	" "			2.00					"
6	" "			4.00					"
7	Dakin's hypochlorite 0.41 per cent (bleaching powder)			0.10	Sodium hypochlorite.		0.40	2.00	Lived.
8	" " 0.41 "			0.50	" "		2.00	10.00	"
9	" " 0.47 "			0.50	" "		2.30	12.00	"
10	" " 0.49 "			0.50	" "		2.40	12.00	"
11	" " 0.50 "			0.50	" "		2.50	12.00	"
12	" " 0.47 "			1.00	" "		4.70	24.00	Died in 15 hrs.†
13	" " 0.50 "			1.00	" "		5.00	25.00	" " 18 " †
14	" " 0.41 "			2.00	" "		8.20	40.00	" " 3 "
15	" " 0.47 "			2.00	" "		9.40	47.00	" " 15 " †
16	" " 0.41 "			4.00	" "		16.40	80.00	" " 1½ "
17	Hychlorite, 0.5 per cent sodium hypochlorite.			0.33	Sodium hypochlorite.		1.70	8.00	Lived.
18	" " 0.5 "			0.33	" "		1.70	8.00	"
19	" " 0.5 "			0.50	" "		2.50	12.00	"
20	" " 0.5 "			0.50	" "		2.50	12.00	Died in 6 hrs.

21	Chloramine-T 0.2 per cent.....	0.10	Sodium hypochlorite.	0.05	0.26	Lived.
22	" 0.2 " "	0.30	" "	0.16	0.80	"
23	" 2.0 " "	0.10	" "	0.50	2.60	Died in 19½ hrs.
24	" 2.0 " "	0.50	" "	2.60	13.00	" " 2 "
25	" 2.0 " "	0.50	" "	2.60	13.00	" " 1½ "
26	" 2.0 " "	0.50	" "	2.60	13.00	" " 2½ "
27	Dichloramine-T 0.5 per cent in bland oil.....	0.10	Sodium hypochlorite.	0.30	1.60	Lived.
28	" 0.5 " " "	0.10	" "	0.30	1.60	Died in 30 hrs.
29	" 0.5 " " "	0.30	" "	0.90	4.70	Lived.
30	" 0.5 " " "	0.30	" "	0.90	4.70	"
31	" 5.0 " " "	0.10	" "	3.10	15.50	Died in 18 hrs.†
32	" 5.0 " " "	0.10	" "	3.10	15.50	" " 18 "†
33	" 5.0 " " "	0.20	" "	6.20	31.00	" " 18 "†
34	" 5.0 " " "	0.20	" "	6.20	31.00	" " 18 "†
35	" 5.0 " " "	0.33	" "	10.60	50.00	" " 2 "
36	" 5.0 " " "	0.50	" "	15.50	75.00	" " 1½ "
37	" 5.0 " " "	0.50	" "	15.50	75.00	" " 1½ "
38	Paraffin oil.....	0.50				Lived.
39	Bland oil.....	0.33				"
40	"	0.50				"
41	Mercuric chloride 0.01 per cent.....	0.10	Mercuric chloride.	0.01	0.05	Lived.
42	" 0.01 " "	0.30	" "	0.03	0.15	"
43	" 0.10 " "	0.10	" "	0.10	0.50	"
44	" 0.10 " "	0.50	" "	0.50	2.50	Died in 18 hrs.
45	Mercuraphen 0.01 per cent.....	0.50	Mercuraphen.	0.05	0.25	Lived.
46	" 0.10 " "	0.10	" "	0.10	0.50	"
47	" 0.10 " "	0.30	" "	0.30	1.50	Died in 48 hrs.
48	" 0.10 " "	1.00	" "	1.00	5.00	" " 27 "

* Cullen, G. E., and Austin, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1917-18, xv, 41.

† Died over night.

TABLE I—Continued.

Animal No.	Solution.	Amount of solution.	Measured in terms of.		Amount of drug.		Amount of drug per 100 gm. of body weight.	Result.
			cc.		mg.			
49	Brilliant green 0.01 per cent.	0.10	Brilliant green.	0.01	0.05	Lived.		
50	" " 0.01 "	0.30	" "	0.03	0.15	Died in 48 hrs.		
51	" " 0.10 "	0.10	" "	0.10	0.50	" " 6 "		
52	" " 0.10 "	0.50	" "	0.50	2.50	" " 18 "		
53	" " 0.10 "	0.50	" "	0.50	2.50	" " 3½ "		
54	Proflavine 0.1 per cent.	0.33	Proflavine.	0.33	1.70	Lived.		
55	" " 1.0 "	0.10	" "	1.00	5.00	" "		
56	" " 1.0 "	0.50	" "	5.00	25.00	Died in 18 hrs.†		
57	" " 1.0 "	0.50	" "	5.00	25.00	" " 12 "		
58	" " 1.0 "	1.00	" "	10.00	50.00	" " 3 "		
59	" " 1.0 "	1.00	" "	10.00	50.00	" " 18 "		
60	Tincture of iodine 7.0 per cent.	0.10	Iodine.	7.00	35.00	Lived.		
61	" " 7.0 "	0.30	" "	21.00	100.00	Died in 45 min.		
62	" " 7.0 "	0.50	" "	35.00	175.00	" " 10 "		
63	" " 7.0 "	1.00	" "	70.00	350.00	" " 15 "		
64	Commercial hypochlorite (Javelle water) 0.5 per cent of sodium hypochlorite.	0.50	Sodium hypochlorite.	2.50	12.00	Lived.		
65	Commercial hypochlorite (Javelle water) 1.0 per cent of sodium hypochlorite.	0.50	" "	5.00	25.00	Died in 4½ hrs.		
66	Commercial hypochlorite (Javelle water) 1.0 per cent of sodium hypochlorite.	1.00	" "	10.00	50.00	" " 2½ "		

67	Peroxide of hydrogen 3.0 per cent (commercial).....	0.50	Peroxide of hydrogen.	15.00	75.00	Lived.
68	Magnesium hypochlorite equivalent to 0.5 per cent sodium hypo- chlorite.....	0.50	Sodium hypochlorite.	2.50	12.00	Lived.
69	Magnesium hypochlorite equivalent to 1.0 per cent sodium hypo- chlorite.....	0.50	"	5.00	25.00	Died in 25 min.
70	Magnesium hypochlorite equivalent to 1.0 per cent sodium hypo- chlorite.....	1.00	"	10.00	50.00	" " 10 "
71	Phenol 0.25 per cent.....	0.50	Phenol.	1.25	6.20	Lived.
72	" 1.00 "	0.30	"	3.00	15.00	"
73	" 1.00 "	0.50	"	5.00	25.00	"
74	" 1.00 "	1.00	"	10.00	50.00	"
75	" 1.00 "	1.00	"	10.00	50.00	Died in 5 min.
76	" 1.00 "	1.50	"	15.00	75.00	" " 12 "
77	Eucalyptol 10.0 per cent in paraffin oil.....	0.10	Eucalyptol.	0.01	0.05	Lived.
78	" 10.0 " "	0.30	"	0.03	0.15	Died in 4 hrs.
79	" 100.0 " "	0.10	"	0.10	0.50	" " 10 min.
80	" 50.0 " " in paraffin oil.....	0.50	"	0.25	1.25	" " 10 "

Sodium hypochlorite equivalent of

Dakin's hypochlorite	= 100	per cent.
Commercial hypochlorite (Javelle water)	= 100	" "
Hychlorite	= 100	" "
Magnesium hypochlorite	= 117.4	" "
Chloramine-T	= 26.5	" "
Dichloramine-T	= 62.1	" "

TABLE II.
Results of Injecting Guinea Pigs Subcutaneously.

Animal No.	Weight.	Solution.	Amount of solution.	Measured in terms of.	Amount of drug.	Amount of drug per 100 gm. of body weight.	Result.
	gm.		cc.		mg.	mg.	
1	400	Dakin's hypochlorite 0.5 per cent.	10.0	Sodium hypochlorite.	50.00	12.00	Lived.
2	550	" " 0.5 " "	13.7	" "	68.00	12.00	"
3	425	" " 0.48 " "	10.6	" "	51.00	12.00	"
4	475	" " 0.5 " "	11.9	" "	60.00	13.00	"
5	450	" " 0.48 " "	22.5	" "	108.00	24.00	Died in 12 hrs.
6	350	Hychlorite, 0.5 per cent sodium hypochlorite...	9.0	Sodium hypochlorite.	45.00	13.00	Lived.
7	450	Chloramine-T 2.0 per cent.	11.25	Sodium hypochlorite.	59.00	13.00	Lived (sloughed).
8	550	" " 2.0 " "	13.7	" "	73.00	13.00	"
9	450	Eucalyptol 50.0 per cent.	11.25	Eucalyptol.	5,600.00	1,200.00	Died in 12 hrs.
10	350	Dichloramine-T 5.0 per cent in bland oil.....	9.0	Sodium hypochlorite.	280.00	83.00	Lived (sloughed).
11	575	Proflavine 0.1 per cent.	7.2	Proflavine.	7.20	1.25	Lived.
12	300	" " 0.1 " "	7.5	"	7.50	2.50	"

TABLE III.
Results of Injecting Guinea Pigs Intraperitoneally.

Animal No.	Weight.	Solution.	Amount of solution.	Measured in terms of.	Amount of drug.	Amount of drug per 100 gm. of body weight.	Result.
	gm.		cc.		mg.	mg.	
13	507	Dakin's hypochlorite 0.5 per cent.....	3.10	Sodium hypochlorite.	15.00	3.00	Lived.
14	350	" " 0.5 " ".....	4.37	" "	22.00	6.30	Died in 9½ hrs.
15	375	" " 0.5 " ".....	9.37	" "	47.00	12.50	" " 7½ "
16	566	Hychlorite, 0.5 per cent sodium hypochlorite.	3.50	Sodium hypochlorite.	17.00	3.00	Lived.
17	500	" " 0.5 " " "	6.25	" "	31.00	6.20	Died in 3¼ hrs.
18	450	" " 0.5 " " "	11.25	" "	56.00	12.50	" " 7½ "
19	430	Chloramine-T 0.2 per cent.	2.70	Sodium hypochlorite.	1.40	0.30	Lived.
20	600	" " 2.0 " "	1.20	" "	6.40	1.10	" "
21	502	" " 2.0 " "	3.10	" "	16.00	3.20	Died in 3 hrs.
22	425	" " 2.0 " "	5.30	" "	29.00	6.70	" " 1½ "
23	450	" " 2.0 " "	11.25	" "	59.00	13.30	" " 1 hr.
24	525	Proflavine 0.1 per cent.	6.55	Proflavine.	7.00	1.30	Lived.
25	525	" " 0.1 " "	13.10	" "	14.00	2.60	" "
26	597	" " 0.1 " "	30.00	" "	30.00	5.00	Died in 72 hrs.
27	470	Dichloramine-T 0.5 per cent in bland oil.	1.90	Sodium hypochlorite.	5.90	1.30	Lived.
28	470	" " 0.5 " " "	5.90	" "	18.30	3.90	Died in 22 hrs.
29	550	" " 5.0 " " "	2.20	" "	68.00	12.30	Lived.
30	498	" " 5.0 " " "	6.20	" "	193.00	39.00	Died in 1½ hrs.
31	375	" " 5.0 " " "	4.70	" "	146.00	39.00	" " 2 "
32	375	" " 5.0 " " "	9.37	" "	292.00	78.00	" " 1 hr.

TABLE IV.

Summary.

Drug.	A. Mice, injected intraperitoneally.		B. Guinea pigs, injected intraperitoneally.		C. Guinea pigs, injected subcutaneously.	
	Smallest fatal dose per 100 gm. of body weight.	Largest surviving dose per 100 gm. of body weight.	Smallest fatal dose per 100 gm. of body weight.	Largest surviving dose per 100 gm. of body weight.	Smallest fatal dose per 100 gm. of body weight.	Largest surviving dose per 100 gm. of body weight.
	mg.	mg.	mg.	mg.	mg.	mg.
Eucalyptol.....	0.15	0.05				
Brilliant green.....	0.15	0.05				
Mercurophen.....	1.50	0.50				
Mercuric chloride.....	2.50	0.50				
Chloramine-T.....	2.60	0.80	3.2	1.1		
Dichloramine-T.....	1.60 (?)		3.9 (?)			
	15.50	4.70	39.0	12.3		
Proflavine.....	25.00	5.00	5.0	2.6		
Hychlorite.....	12.00	12.00	6.2	3.0		
Dakin's hypochlorite.....	24.00	12.00	6.3	3.0	24.0	13.0
Commercial hypochlorite (Javelle water).....	25.00	12.00				
Magnesium hypochlorite.....	25.00	12.00				
Iodine.....	100.00	35.00				
Phenol.....	50.00	50.00				

All the figures represent milligrams of antiseptic, or in the case of the chlorinated antiseptics, milligrams of sodium hypochlorite equivalent of the antiseptic, per 100 gm. of body weight.

Proflavine, hychlorite,⁶ and Dakin's hypochlorite solution given intraperitoneally are all about two or three times as toxic per 100 gm. of body weight for the guinea pig as for the mouse. On the whole, however, the toxicity of the antiseptics follows about the same order in the two species of animal. When the great difference in the body weight of the mouse and the guinea pig is considered, the constancy of the lethal dose per unit of body weight is striking.

While it is, of course, not justifiable to calculate arbitrarily, on the basis of body weight alone, the fatal dose of these substances for man, it is interesting in this connection to note that if such a compu-

⁶ Made by General Laboratories, Madison, Wisconsin.

tation could be considered valid the following amounts of certain of the antiseptics under the proper circumstances would constitute a fatal dose for a man weighing 70 kg.

0.14 cc. of equal parts of paraffin oil and eucalyptol (formerly considerably used as a solvent for dichloramine-T).

144 cc. of a 2 per cent solution of chloramine-T.

160 cc. of a 5 per cent solution of dichloramine-T in bland oil.

1,600 cc. of any of the hypochlorite solutions tested, having sodium hypochlorite titration of 0.5 per cent.

However, only a small amount of the antiseptic employed is absorbed from wound surfaces or from an abscess cavity, and little if any danger from constitutional effects would be expected from their employment in this way. When used in closed cavities, in the serous cavities of the body, or when sutured within a wound, these figures should, we believe, be kept in mind. This is especially the case in respect to eucalyptol used as a vehicle.

The drugs are tabulated in Table IV in the order of diminishing toxicity. It is interesting to note that the least toxic drugs that are efficiently bactericidal are the hypochlorite series and iodine.^{7, 8, 9} The only two of the four hypochlorite solutions studied that are suitable for clinical use are Dakin's hypochlorite solution (in this case made from bleaching powder) and hychlorite. Of the other substances which vary somewhat in their greater toxicity for mice and guinea pigs, the most efficient are proflavine, dichloramine-T, chloramine-T, and possibly brilliant green. Mercurophen, mercuric chloride, and phenol can be disregarded as having too feeble disinfecting powers. Eucalyptol, the most toxic substance included in this study, is not recommended as a bactericidal agent, but merely as a solvent for dichloramine-T. Of these drugs, the only ones having appreciable solvent action on necrotic tissues, pus, etc., are the hypochlo-

⁷ Dakin, H. D., and Dunham, E. K., *Handbook of antiseptics*, New York, 2nd edition, 1918.

⁸ Dakin, H. D., Cohen, J. B., and Kenyon, J., *Brit. Med. J.*, 1916, i, 160.

⁹ Dakin, H. D., Cohen, J. B., Daufresne, M., and Kenyon, J., *Proc. Roy. Soc. London, Series B*, 1916, lxxxix, 232.

rites.^{10, 11} Chloramine-T and the hypochlorites have also a destructive action on bacterial toxins.¹²

CONCLUSIONS.

1. The substances injected intraperitoneally into mice and guinea pigs arranged in the order of their decreasing toxicity are: eucalyptol and brilliant green; mercuraphen; mercuric chloride and chloramine-T; dichloramine-T and proflavine; hychlorite, Dakin's hypochlorite, Javelle water, and magnesium hypochlorite; iodine and phenol.

2. Now that Dakin's bland solvent, chlorcosane, is available as a vehicle for dichloramine-T, eucalyptol should probably be discarded for this purpose because of its much greater toxicity.

3. Inasmuch as experienced surgeons do not approve of the injection of solutions of iodine and phenol into closed cavities, it would seem advisable not to use any of the antiseptics here discussed in this manner inasmuch as all exhibit a greater toxicity for mice and guinea pigs than the two chemicals first named.

4. The method of testing toxicity of antiseptics by subcutaneous injection is not satisfactory because exudation and subsequent sloughing reduce the rate of absorption and make uncertain the amount finally absorbed.

¹⁰ Taylor, H. D., and Austin, J. H., *J. Exp. Med.*, 1918, xxvii, 155.

¹¹ Austin, J. H., and Taylor, H. D., *J. Exp. Med.*, 1918, xxvii, 627.

¹² Taylor, H. D., and Austin, J. H., *J. Exp. Med.*, 1918, xxvii, 375.

THE SPIROCHETAL FLORA OF THE NORMAL MALE GENITALIA.

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PLATES 30 TO 32.

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An accurate knowledge of the varieties of spirochetal organisms which normally inhabit the smegma or the mucous membranes of the urogenital region has become imperative for the establishment of an etiological relationship between a spirochete and a disease in which the organism may be found in the urine.

The classic work of Inada, Ido, Hoki, and others¹ on the presence of *Leptospira icterohæmorrhagiæ* in the urine of convalescents from infectious jaundice has introduced a new procedure by which the disease may be easily diagnosed, and it is natural that a similar procedure should be followed in the search for an etiological agent in other diseases of infectious origin.

Attention has been directed by Martin,² Nankivell and Sundell,³ and Patterson⁴ to the urine in cases of trench fevers. In fact, Nankivell and Sundell early demonstrated minute spirochetes in specimens of urine from soldiers suffering from so called trench fever. Of 26 patients, most of them suffering from a "five-day fever," 99 specimens were examined, with 29 positive findings. Spirochetes were found in 12 out of 15 typical cases, while none of the 8 controls showed a spirochete (Fig. 32). The investigators considered the possibility of contamination of the urine from the smegma or from preputial sources, but it seemed to

¹ Inada, R., Ido, Y., Hoki, R., Kaneko, R., and Ito, H., Etiology, mode of infection, and specific therapy of Weil's disease (spirochætosis icterohæmorrhagica), *J. Exp. Med.*, 1916, xxiii, 377.

² Martin, C. J., quoted by Nankivell and Sundell.³

³ Nankivell, A. T., and Sundell, C. E., On the presence of a spirochæte in the urine in cases of trench fever, *Lancet*, 1917, ii, 672.

⁴ Patterson, S. W., Preliminary note on spirochætes occurring in the urine in cases of "P. U. O.," *J. Roy. Army Med. Corps*, 1917, xxix, 503; Spirochætes occurring in the urine in cases of "pyrexia of unknown origin," *Brit. Med. J.*, 1917, ii, 418.

them improbable that the spirochetes came from these sources, since the urine usually contained the minute spirochetes unaccompanied by the coarse *Spiro-nema refringens*, and in case of contamination the presence of the large varieties was to have been expected. Moreover, in some positive instances no spirochetes could be discovered in the smegma. The occurrence of spirochetes in the urine was not constant, that is, not detectable on successive occasions, but was recurrent at irregular intervals. A certain relation seemed to exist between the appearance of spirochetes in the urine and the height of pyrexia, spirocheturia occurring usually 24 hours after the height of fever. In still other cases they appeared on the 14th to the 16th day. They were actively motile, averaged 8.15μ in length and 0.3μ in width, with an average of five curves; *i.e.*, varying from two and a half curves in 5μ to ten curves in 12.5μ . The spirals varied in depth. The extremities tapered to sharp points with a flagellum at one or both ends. The organism differed from *Treponema pallidum* in its shortness and its fewer spirals.

Patterson, using the Fontana, Wilmaers-Renaux,⁵ and India ink methods, examined specimens of urine from various groups of trench affections, 3 cases of trench nephritis, 1 case of pyelonephritis, with abscess of the lungs, 15 cases of relapsing type of pyrexia of unknown origin, 1 case of myalgia following pyrexia of unknown origin, and 5 cases of appendicitis (?) not yet diagnosed, finding spirochetes with the following features: They were about one to one and a half times the diameter of a red blood corpuscle, very thin, with tapering extremities, some having five to eight more or less regular curves, some being straight, some bowed, or lying in a semicircle (Fig. 33). The spirals were not so fine as those of *Treponema pallidum* or so coarse as those of *Spiro-nema recurrentis*. The organisms took Giemsa's, Leishman's, or Romanovsky's stain poorly but were easily demonstrated by the Indian ink method. Patterson depicts the spirochetes found in the abdominal type of cases as rather closely wound, short, thick forms, and those found in the relapsing type as much more tightly coiled. Little attention was given to control cases.

The main objection to the work of the British investigators has been the possibility of an accidental contamination of the urine by unclean surroundings. Many have insisted upon the necessity of collecting specimens by catheterization. The investigation of Stoddard⁶ brought out an unsuspected source of spirochetes in the periurethral as well as the intraurethral region of the male genitalia. After examining 50 healthy soldiers and 50 miscellaneous hospital patients without history or symptoms of relapsing fever (trench fever), Stoddard drew the conclusion that (1) spirochetes are not uncommon organisms in the urethra of men without history or symptoms of relapsing fever; (2) many dif-

⁵ Wilmaers, L., and Renaux, E., Quarante-sept cas de Spirochétose ictero-hémorragique, *Arch. méd. Belges*, 1917, lxx, 115, 207.

⁶ Stoddard, J. L., Occurrence of spirochetes in the urine, *Brit. Med. J.*, 1917, ii, 416.

ferent varieties are found; (3) some of the varieties seen are morphologically closely similar to pathogenic varieties; (4) the spirochetes occur so definitely within the urethra that they are an obvious source of contamination in uncatheterized specimens of urine; (5) they are a sufficiently dangerous source of error even in catheterized specimens to deserve attention in careful work; and that finally (6) it is possible that a staining reaction or some other morphological character may be discovered to differentiate microscopically the common and harmless from the pathogenic spirochetes. Of 50 hospital cases 56 per cent showed spirochetes, of which 46 per cent were not *Spironema refringens*. Of 50 American soldiers spirochetes in the urethra occurred in 22 per cent, 2 per cent of which showed *Spironema refringens* also. Films from periurethral parts contained more of the coarse *refringens* type.

The spirochetes found by Stoddard measured from 3.75 to 22 μ , more commonly 6.75 to 9 μ , but 11 μ was not uncommon. They were either moderately thick or extremely slender. The ends tapered or were blunt and formed a hook. The spiral length varied from 0.5 to 1 μ . Flattened and longer spirals also occurred, averaging 1.5 to 3.3 μ and as long sometimes as 4 μ . Stoddard states that a type with about eleven curves in 7.5 μ occurs frequently, but I have not been able to verify this finding. In some cases the spirals were exceedingly close and fine and almost impossible to count. They were often irregular, the deep narrow, deep wide, flat narrow, and flat wide types of spirals sometimes occurring simultaneously in the same organism. The middle portion sometimes had looser coils or none at all. In one film many different varieties were often present, including frequently organisms similar to *Leptospira icterohæmorrhagiæ*.⁷

Obviously it is not simple to interpret what one sees in the rich spirochetal material as described by Stoddard, who sees in it many different varieties, including the leptospira type. But, as this article is intended to show, a critical analysis of the spirochetal flora reduces the number of varieties to not more than three, or at most four; namely, *Spironema refringens*, *Treponema calligyrum*, and *Treponema minutum*, n. sp.

Since the time of Schaudinn and Hoffmann a coarse spirochete designated by them *Spirochæta refringens* has been known to inhabit the genital region, but no particular attention has been given to the possibility of the existence of other varieties. It was not until the subject was taken up not only from the morphological but also from the cultural standpoint that some interest came to be attached to these

⁷ Noguchi, H., *Spirochæta icterohæmorrhagiæ* in American wild rats and its relation to the Japanese and European strains. First paper, *J. Exp. Med.*, 1917, xxv, 755.

spirochetes. In the present work a strain of *Spirocheta refringens* was isolated and its morphological and cultural features studied, thus establishing its entity as a species. Later a strain of spirochete (*Treponema calligyrum*) was obtained from a condyloma, which resembled *Treponema pallidum* on the one hand and *Spirocheta refringens* on the other, being an intermediary organism in its morphological and cultural characteristics. Subsequent observations have led me to regard this particular species as one of the most common varieties that are found in the flora of the smegma or of the urethral region. In fact, *Treponema calligyrum* is more frequently met with than the better known coarse *Spirocheta refringens*. There is, in addition to these two varieties, another, much smaller spirochete in the genital flora, which will be described in a subsequent paragraph. These three, the minute, medium, and coarse types, constitute the spirochetal flora which at first glance present such a complex aspect.

The smegma and urethral films from six soldiers who were admitted to the Hospital of The Rockefeller Institute for treatment for pneumonia have been examined.⁸ The specimens were examined in fresh condition under the dark-field microscope and also as stained preparations. For staining methods Giemsa's stain, Fontana's silver impregnation,⁹ and occasionally Benians' Congo red negative im-

⁸ These specimens were obtained through the courtesy of Captain Henry T. Chickering.

⁹ (a) Fix the air-dried film in Solution 1, which consists of

	cc.
Glacial acetic acid.....	8
Formalin.....	20
Distilled water.....	100

for 1 minute and wash well with water. (b) Mordant with Solution 2, which consists of

Tannin.....	5 gm.
Phenol.....	1 cc.
Water.....	100 "

for 1 minute over a gentle flame to the point of steaming, then wash thoroughly in water. (c) Treat in a 0.25 per cent silver nitrate solution to which one drop of ammonia is added to 40 cc. of the solution. The film turns brown in a few minutes. Wash in water and then (d) cover with the mordant and warm it over a flame until it begins to steam. Then wash the film in water and dry.

pression method were employed;¹⁰ also a mordant staining recommended by me for various spirochetes, including *Treponema pallidum*. The method is similar to that advanced by Wilmaers and Renaux, but seems to give a better color value on account of the use of gentian violet instead of fuchsin. The film is fixed in methyl alcohol for 15 minutes, then after being washed in water is covered with a solution of mordant (5 per cent tannin plus 1 per cent phenol) and held over a gentle flame for 1 minute, during which time it begins to steam. It is again washed in running water, covered with a strong aqueous solution of gentian violet to which 1 per cent phenol has been added, and steamed briefly over a flame, then washed well in water, air-dried, and examined. This method gives excellent results also with the *pallidum*. Care must be taken not to make too thick a film.

The number of cases examined was small, but the finding was such that it was sufficient to determine the average flora in male genitalia. The varieties of spirochetes encountered in most of the smegma were the same as those found in one. All contained *Treponema calligyrum* and *Treponema minutum*, *n. sp.*,¹¹ and most of them *Spironema refringens*, although the latter was absent in some cases.

No spirochetes were found in the films made from the urethral mucosa by means of a platinum loop. Just where the fault in the technique lay I am unable to explain. The finding was uniformly negative also with the specimens of urine from ten soldiers. With the idea that in nephritis cases there might be more possibility of encountering spirochetes in the urine, ten different specimens from acute as well as chronic cases of nephritis were subjected to a careful examination, but with no positive finding as yet.¹² In Table I

¹⁰ A few drops of a 2 per cent Congo red solution (filtered) are mixed with a drop of the material suspected of containing a spirochete and spread over a clean slide to form a film. The slide after being air-dried is immersed in a jar of absolute alcohol containing 1 per cent hydrochloric acid. In a few minutes the red color of the film turns to a bluish tint. The slide is then removed from the acid alcohol and air-dried.

¹¹ Noguchi, H., Morphological characteristics and nomenclature of *Leptospira* (*Spirochæta*) *icterohæmorrhagiæ* (Inada and Ido), *J. Exp. Med.*, 1918, xxvii, 575.

¹² The specimens used in these tests were obtained through the courtesy of Dr. W. W. Palmer of the Presbyterian Hospital.

are recorded some of the results obtained in the present study. There are at least three different varieties distinguishable in the photomicrographs or under the dark-field microscope, a minute (*minutum*), a medium (*calligyrum*), and a large (*refringens*) type. Their biometric characteristics, as encountered in twenty-five specimens of each type, are given in Table I.

TABLE I.

Type.	Length.		Thickness.	Spiral amplitude and intervals.	Spiral depth.	No. of spirals or waves.
	Average.	Extremes.				
Minute type.	7-10 μ	3-14 μ	0.25-0.3 μ	0.9-1 μ . Fairly regular intervals.	0.2-0.5 μ . Some may reach 1 μ in penultimate spirals.	7-10 spirals; vary according to length.
Medium type.	9-12 μ	4-14 μ	0.35-0.4 μ	1.75 μ . Usually fairly regular; that is, a given amplitude is well maintained in a specimen.	0.5-1 μ . Often flattened near the middle in stained specimens. Reaches 1.5 μ in some.	5-8, varying according to the spiral amplitude; some only 3.
Large type.	12-16 μ	7-22 μ	0.7 μ	2-3 μ . Usually more or less regular.	0.5-1.5 μ . Almost constant; in live specimens changing the position of the waves. In stained specimens often irregularly flattened out.	3-5; quite variable; exceptionally 8 in a very long specimen.
<i>T. pallidum</i> .	8-14 μ	6-18 μ	0.25-0.3 μ	1 μ	0.8-1 μ	8-14; some 16.

Some of the dark-field, as well as the ordinary photomicrographs, representing the minute, the medium, and the large types are shown in Figs. 1 to 14. The minute type is decidedly smaller than *Treponema pallidum* and has a larger number of shallower spirals in proportion to its length (Figs. 1 and 5). There are also many short specimens such as are never found among the *pallida*. The medium type has an aspect like that of atypical specimens of the *pallidum*. The spirals are fairly deep but not so deep as those of a typical *pallidum*, while the intervals between them are wider (Figs. 2, 5, 6, 9, and 10). All appear somewhat thicker than the *pallidum* (Fig. 21) when seen under the dark-field microscope. This does not apply to the specimens stained by mordanting techniques (Fontana's and the writer's), in which there occurs often an uncontrollable uneven heavy deposit of the dyes, due to various external factors (Figs. 5 to 14, 17, 20, and 22). Among organisms of the medium type are noticed two forms, one with more closely set spirals and the other with wider ones, but this is due to certain temporary conditions and may be made to disappear or reappear by regulation of cultural conditions. For example, there will be more of the wide, flat spiral forms when the medium is more fluid. The large type is much heavier, comparatively short, with few spirals, and constantly changes its curves (Figs. 4 and 8). The spirals of the minute type become readily obliterated after the death of the organism (upper organism in Fig. 8).

When fresh they all exhibit moderately active movements, rotary, lashing, and forward and backward locomotion. The large type is the most energetic and the minute variety the least so. In many of the large type there is a distinct double contour effect upon examination under the dark-field microscope. All are provided with a terminal filament or flagellum at one or both ends.

As has been noted before, not all smegmata contain a spirochete, and the varieties present may all belong to one or two of the three groups. As a rule, however, all three types are present, the medium type usually predominating.

Cultural Characteristics.

By selecting the smegma specimens which were rich in the type desired, a culture of each of the three types described was obtained. The technique employed was similar to that previously used for the cultivation of *Spironema refringens*¹³ and *Treponema calligyrum*.¹⁴ All require strict anaerobiosis (addition of fresh tissue to the media), the presence of suitable body fluid (ascitic fluid), and an optimal temperature (37°C.). The growth in the fluid medium, consisting of ascitic fluid and a piece of fresh rabbit kidney and a layer of paraffin oil, is invisible, while in a solid medium, consisting of 2 parts of the neutral agar and 1 part of ascitic fluid with a piece of the fresh rabbit kidney at the bottom, a faint haze appears to develop near the tissue, gradually extending upward within a fortnight. No discrete, circumscribed, sharp colonies have so far been observed. In this respect all the strains obtained are analogous to the cultures of various anaerobic treponemata and spironemata.¹⁵ None produced a putrefactive or offensive odor, the absence of odor from the culture of the minute type serving to distinguish it from either *Treponema microdentium*¹⁶ or *Treponema mucosum*.¹⁷ Carbohydrates added to the culture media exert neither a favorable nor a retarding influence upon growth, and no visible alterations of the media result from their presence.

In young fluid cultures, whether of the minute, medium, or large type, the organisms are short and active, but as they grow older (2 weeks) the longer forms, some in chains, and some in tangled masses, predominate, their motility meanwhile being considerably reduced. The spirals are quite regular (Figs. 23 to 31). Very short forms do

¹³ Noguchi, Pure cultivation of *Spirochæta refringens*, *J. Exp. Med.*, 1912, xv, 466.

¹⁴ Noguchi, Cultivation of *Treponema calligyrum* (new species) from condylomata of man, *J. Exp. Med.*, 1913, xvii, 89.

¹⁵ Noguchi, Experimental research in syphilis with especial reference to *Spirochæta pallida* (*Treponema pallidum*), *J. Am. Med. Assn.*, 1912, lviii, 1163.

¹⁶ Noguchi, Cultural studies on mouth spirochætæ, *Treponema microdentium* and *macrodentium*, *J. Exp. Med.*, 1912, xv, 81.

¹⁷ Noguchi, *Treponema mucosum* (new species), a mucin-producing spirochæta from pyorrhea alveolaris, grown in pure culture, *J. Exp. Med.*, 1912, xvi, 194.

not appear in the solid media, the organisms appearing to attain average length within a short time. The spirals are remarkably regular in solid media and so deep, in the case of the medium type, as to simulate a *pallidum* (Fig. 29). In older cultures two, three, and four individuals in chains have occasionally been encountered (Fig. 27). Division in all three types is brought about by transverse and perhaps also by longitudinal fission.

Identification.

The morphological and cultural characteristics of the large type show it clearly to be a *Spironema refringens*, those of the medium type identify it with *Treponema calligyrum*. The latter type may be the same organism as that described by Levaditi and Stanesco¹⁸ in 1909 as *Spirochæta gracilis*, found in a case of balanitis, but, as pointed out previously, these authors used a name already designating another spironema from an ulcerating jaw, which is very different from the present medium type. The name *Treponema calligyrum* was given to a non-pathogenic spirochete cultivated from the surface of a condyloma, but subsequent studies on the spirochetal flora of the genitalia have convinced me that this type is one of the most commonly met inhabitants of the genital region.

The minute type is not unlike the minute spirochete of the mouth, *Treponema microdentium*, but its cultural characteristics differentiate it from the latter. *Treponema microdentium* produces a peculiar odor, especially when freshly isolated, and in a fluid medium the color of the fresh tissue is made grayish within about 10 days and the fluid somewhat faintly opalescent. The *minutum* produces no odor and remains without any perceptible action upon the culture medium, though in dimension there is a general resemblance.

In order to determine whether these two closely similar organisms are immunologically related to each other, agglutination tests were undertaken in which the action of a *microdentium* antiserum (rabbit) was tested on both types. It was found that the serum caused a marked agglutination of *Treponema microdentium* in 1:500 dilution but only a slight one with two different strains of the *minutum*, even

¹⁸ Levaditi, C., and Stanesco, V., Culture de deux spirochètes de l'homme *Sp. gracilis* et *Sp. balanitidis*, *Compt. rend. Soc. biol.*, 1909, lxxvii, 188.

in a dilution of 1:20. In this connection it may be mentioned that a *calligyrum* serum (rabbit) gave a copious agglutination with the cultures of the medium type in a 1:200 dilution, but only a slight one with those of the minute type in 1:20. There was a partial reaction, but not marked enough to render the differentiation of the two types difficult.

In all probability the minute smegma spirochete has been repeatedly observed by investigators, but no special attention seems to have been given to its identity. I have been accustomed to pass it over as probably identical with *Treponema microdentium*. Now that this type has been found to constitute an independent group, differentiated by several well defined features, it may well be known under a separate name, *Treponema minutum*.

In the spirochetal flora of male smegma, therefore, only the three forms, *Spironema refringens*, *Treponema calligyrum*, and *Treponema minutum*, were recognized.

DISCUSSION AND SUMMARY.

The varieties of spirochetes enumerated and photomicrographed from the male smegma flora represent practically every form hitherto described by Nankivell and Sundell and by Patterson in the specimens of urine from trench fever cases (Figs. 32 and 33). The urethral flora, as studied by Stoddard, seem to contain more varieties, but, except those of his more detailed morphological descriptions, every form observed by him is among those found in the smegma. Stoddard saw certain forms with hooked ends suggestive of the *Leptospira icterohæmorrhagiæ* of infectious jaundice, but the resemblance ends with this one feature, and differentiation should always be possible under the dark-field microscope, by means of which the leptospira reveals its highly characteristic minute elementary spirals, presenting the appearance of a chain of dots (Fig. 18). Fig. 19 shows that a very favorable fixation with the osmic acid vapor followed by Giemsa's staining may also bring out the elementary spirals. Of all the spirochetes, none has so closely set spirals as the jaundice leptospira, the distance between two spirals being only 0.5μ . Various methods, including Fontana's, Benians', the mordant gentian violet stain, or Burri's India ink method, are inadequate

to differentiate the leptospira from other spirochetes (Figs. 12, 14, 15, 16, 17).

Why a positive spirochete finding with the films from the urethra and in the specimens of urine was not obtained, is difficult to explain, except on the grounds of the paucity of specimens examined. At all events, the recent negative results reported by Fiessinger¹⁹ with French soldiers and invalids after cleansing of the urethra and glans seem to be in harmony with my results.

In conclusion it may be stated that *Spironema refringens*, *Treponema calligyrum*, and *Treponema minutum* represent practically all the spirochetel forms observed in the male smegma flora. A leptospira has never been conclusively shown to be present in the specimens of normal urine or smegma. For the satisfactory microscopic demonstration of a leptospira a dark-field illuminator is indispensable.

EXPLANATION OF PLATES.

PLATE 30.

Magnification, $\times 1,000$.

FIGS. 1 to 4. Dark-field views of the spirochetes in a male smegma. Fig. 1 represents *Treponema minutum*, Fig. 2 *Treponema calligyrum*, Fig. 3 *Spironema refringens*, and Fig. 4 a *Spironema refringens* (below) and a *Treponema minutum*.

FIGS. 5 to 11. Various types of spirochetes in smegma, stained by Fontana's method.

FIG. 5. Two specimens of *Treponema minutum*.

FIG. 6. A specimen of *Treponema minutum* and two of *Treponema calligyrum*, of varying lengths.

FIG. 7. A group of *Treponema calligyrum*, with two specimens of *Treponema minutum*.

FIG. 8. A group of *Spironema refringens* from a sample of male smegma.

FIGS. 9 to 11. *Treponema calligyrum* from two different specimens of male smegma.

FIGS. 12 and 13. *Treponema calligyrum* in preparation stained by the mordant gentian violet method. In Fig. 12 there are two specimens without distinct spirals which closely resemble *Leptospira icterohæmorrhagiæ* in similar stained preparations. Further study by means of a dark-field microscope is necessary to determine whether they are leptospira or *calligyrum*.

FIG. 14. A group of *Treponema calligyrum* type from a specimen of male smegma, stained by Giemsa's method. The organisms appear much thinner here than in specimens stained by other methods. A hooked spirochete resembling strongly the leptospira is seen near the left upper corner.

¹⁹ Fiessinger, N., À propos des Spirochètes du méat et de l'urine de l'homme normal, *Compt. rend. Soc. biol.*, 1918, lxxxi, 38.

FIGS. 15 to 19. *Leptospira icterohæmorrhagiæ* under various conditions (for comparison).

FIG. 15. Four specimens of *Leptospira icterohæmorrhagiæ* stained by the mordant gentian violet method. They appear blunt and curved and without any indication of the minute elementary spirals which are the characteristic feature of this genus. As they appear here they are indistinguishable from the stretched forms of the *calligyrum* type.

FIG. 16. A few leptospiræ as demonstrated by Benians' Congo red method. Here, too, they do not show their elementary spirals.

FIG. 17. A group of *Leptospira icterohæmorrhagiæ* from a culture, stained by Fontana's method. They fail to show their elementary spirals by this staining.

FIG. 18. A leptospira viewed under the dark-field microscope, showing its minute elementary spirals.

FIG. 19. A number of *Leptospira icterohæmorrhagiæ*, fixed with osmic acid vapor and stained by Giemsa's stain, showing the elementary spirals.

FIGS. 20 to 22. *Treponema pallidum* under different conditions (for comparison).

FIG. 20. *Treponema pallidum* when stained by the mordant gentian violet method.

FIG. 21. *Treponema pallidum* under the dark-field microscope.

FIG. 22. *Treponema pallidum* as stained by Fontana's silver impregnation method.

PLATE 31.

Magnification, $\times 1,000$.

FIGS. 23 and 24. Dark-field view of a culture of *Treponema minutum* from a male smegma.

FIG. 25. *Treponema minutum* from a culture. Stained by the mordant gentian violet method.

FIG. 26. Similar specimens stained by Fontana's method.

FIG. 27. Dark-field view of a culture of *Treponema calligyrum* from a male smegma.

FIG. 28. A culture of *Treponema calligyrum* stained by the mordant gentian violet method.

FIG. 29. Similar specimens stained by Fontana's method.

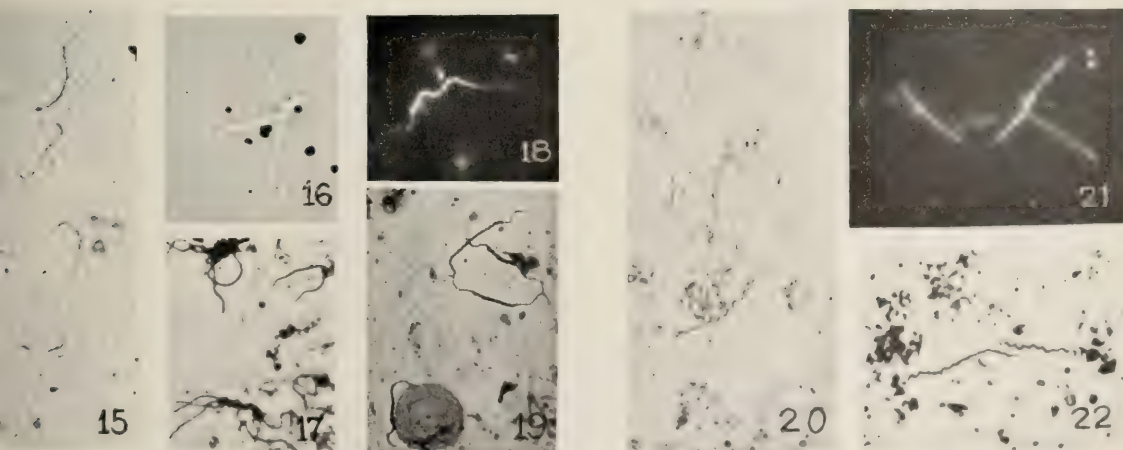
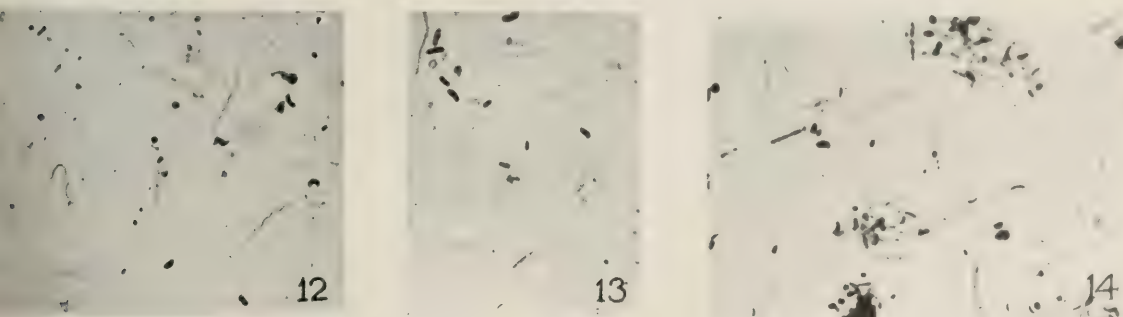
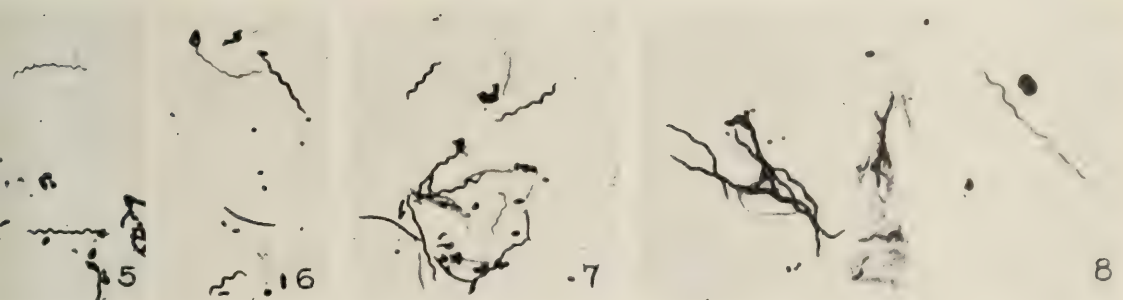
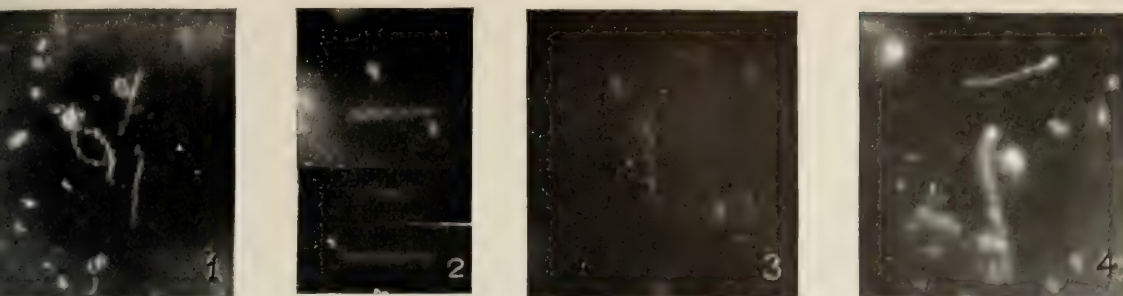
FIG. 30. Dark-field view of a culture of *Spirocheta refringens* from a male smegma.

FIG. 31. Similar specimens stained by Fontana's method.

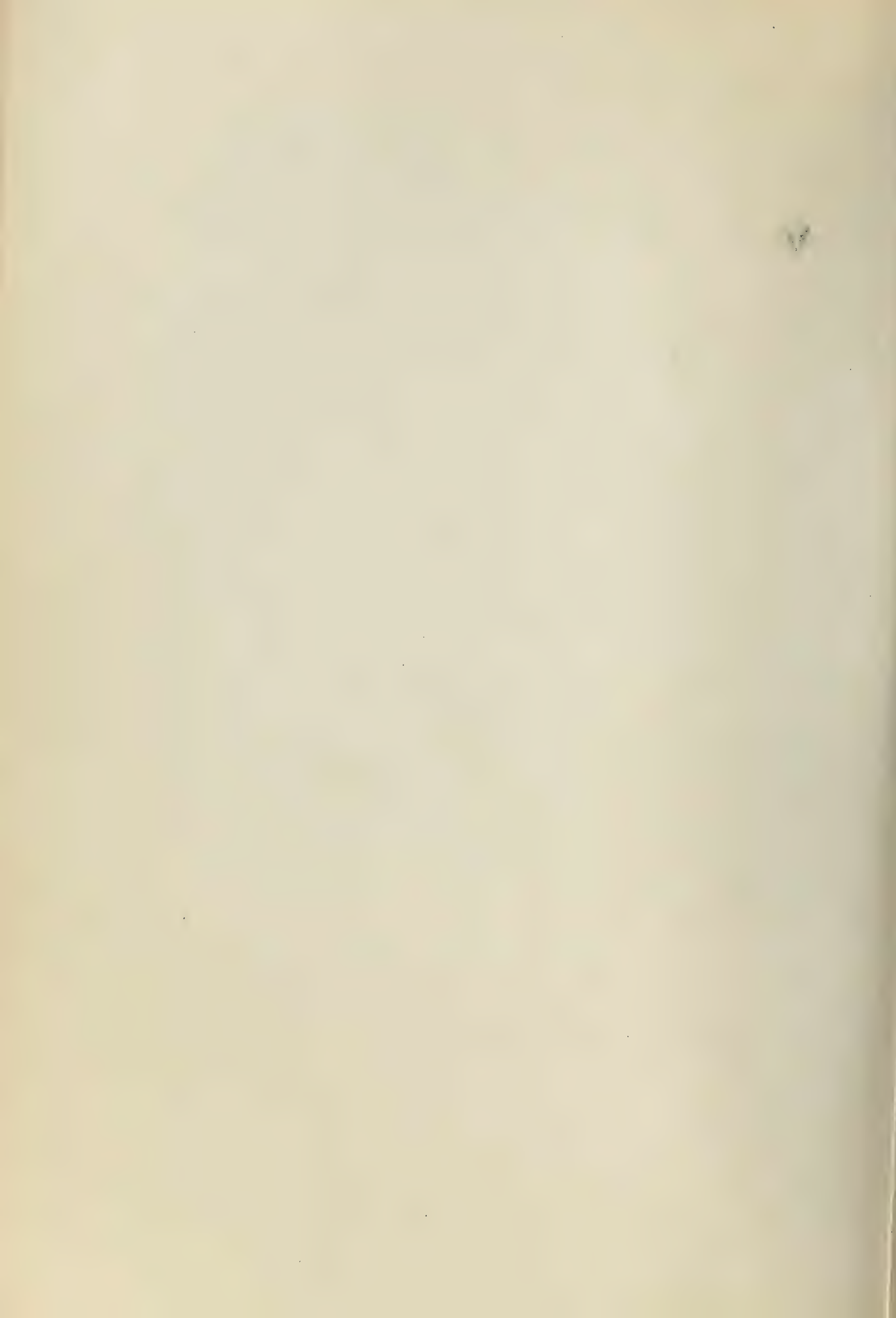
PLATE 32.

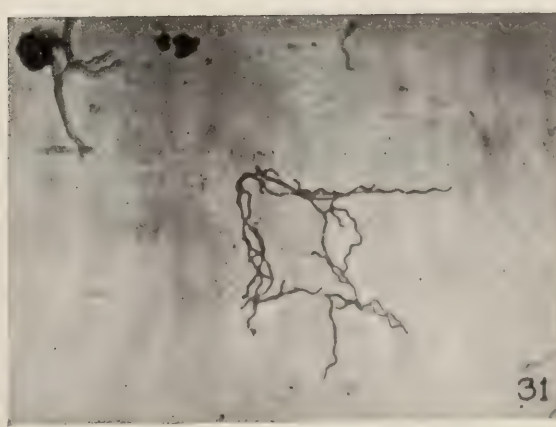
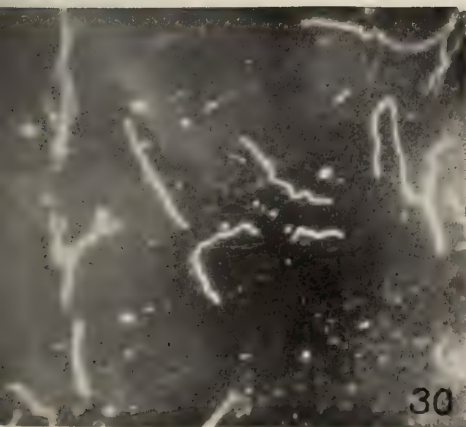
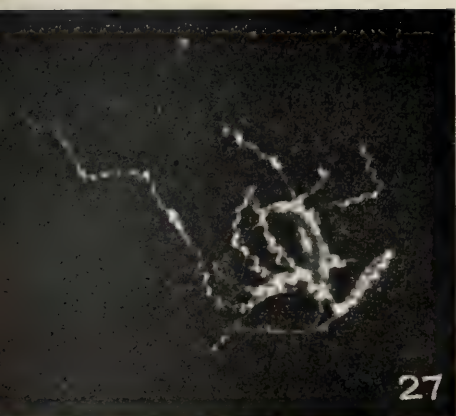
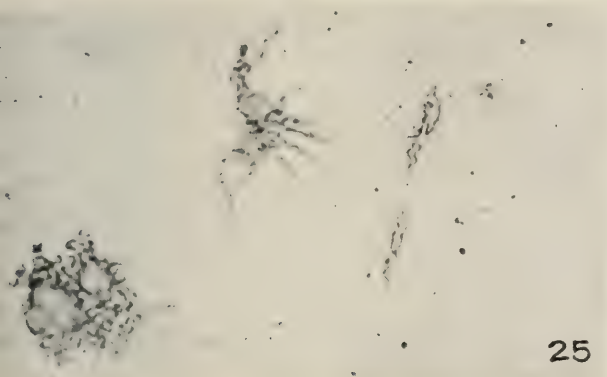
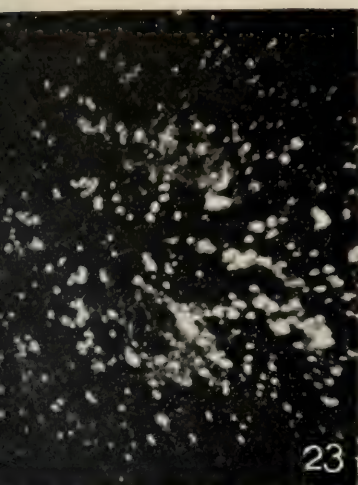
FIG. 32. Photographic reproduction of the photomicrographs of spirochetes in Nankivell and Sundell's article on the spirochetes in the urine in trench fever cases.³

FIG. 33. Photographic reproduction of the schematic drawings by Patterson in his article.⁴



(Noguchi: Spirochetal flora of normal male genitalia.)





(Noguchi: Spirochetal flora of normal male genitalia.)

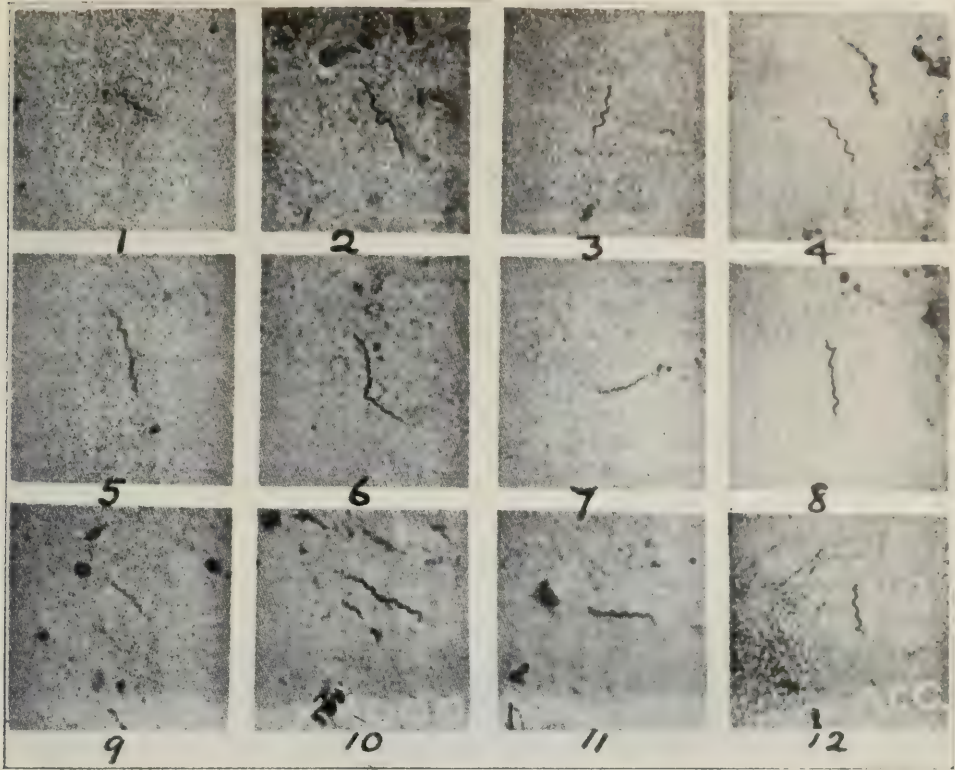
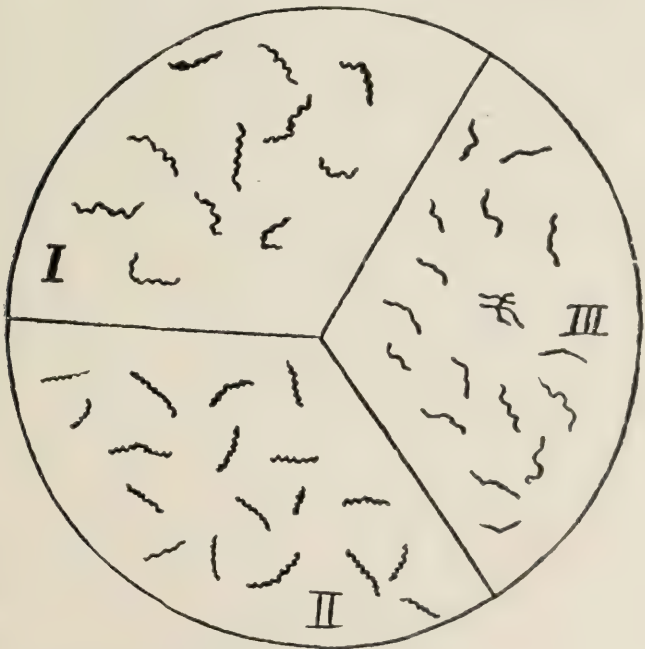


FIG. 32.



- I. Spirochetes of Type 1, abdominal P. U. O.
- II. Type 2, relapsing P. U. O.
- III. Spirillar form from urethra.

FIG. 33.

(Noguchi: Spirochetal flora of normal male genitalia.)

PHYSIOLOGICAL STIMULATION OF THE CHOROID PLEXUS AND EXPERIMENTAL POLIOMYELITIS.

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When the active filterable virus of poliomyelitis is injected into the blood of monkeys, infection and paralysis almost never follow even when the quantity of virus introduced is very large. The reason assigned for the non-infectiousness of the virus under these conditions, compared with the remarkable activity displayed by it when brought into immediate proximity with the central or even the peripheral nervous organs, is the inability of the virus to pass the barrier of the choroid plexus and the blood vessels of the central nervous system. Flexner and Amoss¹ have shown in several series of experiments that when sterile irritating chemical substances are introduced from without and by lumbar puncture into the subarachnoid space, the injury inflicted upon the choroid plexus and blood vessels of the meninges and possibly those of the central nervous organs also, facilitates the passage of the virus from the blood into the nervous tissues under conditions leading to infection, paralysis, and death from poliomyelitic disease. Their experiments have led them to view the meningeal-choroidal complex as constituting in man a defensive mechanism against infection with the virus of poliomyelitis.

According to this view, disturbance of the integrity of the defensive complex arising from any cause would predispose to infection with the virus, provided the disturbance synchronized with the wide distribution of the virus, such as is believed to be the case during epidemics of poliomyelitis. The experiments of Flexner and Amoss have indicated that the qualitative changes in the meningeal-choroidal com-

¹ Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1914, xx, 249; 1917, xxv, 525. Amoss, H. L., and Ebersson, F., *ibid.*, 1918, xxvii, 309.

plex, permitting the escape of the virus from the blood into the nervous tissues, may be almost infinitesimally small. Thus the mere substitution of the cerebrospinal fluid of one monkey for that of another sometimes suffices to open this way. The structural alterations induced by this procedure must be so slight as to be regarded merely as molecular; and yet they have proved adequate to overcome the defensive mechanism.

All the means employed up to the present to disturb the mechanism may be regarded as organic in their effect. Moreover, they have always acted from without, in the sense that they have been brought into relation with the meningeal-choroidal complex through the medium of the cerebrospinal fluid already present in the subarachnoid space. The question presented itself whether a functional effect merely and acting, as it were, from within, might likewise open the way for the passage of the virus from the blood into the nervous tissues. A method was at hand to test experimentally this possibility.

The cerebrospinal fluid is a secretion derived from the blood through the mediation of the choroid plexus. The secreting cells of the plexus exercise a highly precise discrimination in respect to the quality and quantity of the constituents taken from the blood and passed on to the subarachnoid space. The composition of the cerebrospinal fluid not only differs markedly from that of the blood, but dissolved drugs and bacteria and their toxic and other metabolic products present in the blood are capable of being excluded perfectly from the cerebrospinal fluid by the choroidal mechanism.

The stimulus on which the secretory activity of the choroid plexus depends has been shown by Dixon and Halliburton² to be a hormone contained within the choroid plexus and to a less extent in the brain substance. The liberation of this hormone into the blood is the precursor to and regulating medium of the choroidal secretory activity, through which the cerebrospinal fluid is elaborated. By increasing experimentally the quantity of the hormone within the blood the amount of cerebrospinal fluid secreted within a unit of time may be increased.

²Dixon, W. E., and Halliburton, W. D., *J. Physiol.*, 1910, xl, p. xxx; 1913-14, xlvii, 215; 1914, xlviii, 128.

This phenomenon provides, therefore, a means by which the choroid plexus may be stimulated from within and made to perform its secretory function in an intensified manner. It furnishes a simple method for determining whether merely increased functional activity, independent of structural or organic alterations, suffices to open the way for the passage of the poliomyelitic virus from the blood into the central nervous organs under circumstances leading to infection.

The experiments to be described were carried out to determine this point. The procedure followed for preparing and injecting the extract of the choroid plexus was closely modelled on that of Dixon and Halliburton. These investigators found that an intravenous injection of a saline extract of the choroid plexus, after a delay of a few seconds, causes the cerebrospinal fluid to flow actively for a variable time, after which the flow ceases gradually. The second injection produces little or no effect unless the intervening interval of time is about 10 or 15 minutes.

EXPERIMENTAL.

The starting-point of our experiments was a repetition of the decisive experiments of Dixon and Halliburton. The first step was the preparation of an extract of the choroid plexus. 1 gm. of the dried plexus was ground up with clean sand in a mortar in 100 cc. of isotonic saline solution. The suspension was filtered and the filtrate employed for injection. 5 cc. of the filtrate caused a marked increase in the flow of cerebrospinal fluid in a dog weighing 12 kilos. Extracts of the fresh plexus give an equivalent result, and boiling does not destroy the activity. The active substance is, moreover, soluble in dilute and absolute alcohol. An extract of the brain is less active than one of the plexus. Material from the dog, sheep, or ox may be employed. Dixon and Halliburton, who established these points, express the view that some product of the brain's metabolism passes to the choroid plexus and this hormone stimulates to activity the secreting epithelium covering the plexus. They also discuss the possibility of the hormone's originating in the choroidal epithelium and passing secondarily to the brain tissue. They incline to the first alternative.

Preparation of the Choroid Extract. Isotonic.—Plexuses removed from the fresh brains of sheep under sterile conditions are washed free from blood in sterile saline solution, dried between filter paper, weighed, made up to 1 per cent suspension in isotonic saline solution, ground with sand, and filtered.

Hypertonic.—The same steps are followed except that 10 per cent of the dried plexuses are suspended in 8.5 per cent saline solution. The stock solution is then diluted 1 part to 9 of sterile distilled water before injecting. The filtering of the viscous mixture, which is a slow process, may be substituted by rapid centrifugalization. The clear supernatant fluid is removed and diluted as indicated.

Preliminary Tests.

The technique of the experiments was perfected on dogs in accordance with the method devised by Dixon and Halliburton. A single protocol is appended to illustrate a successful experiment.

Oct. 22, 1917. Dog; weight 12.5 kilos. Anesthesia: chloroform, morphine, and urethane (subcutaneous). Subcerebellar cistern punctured. After the first rapid rush of cerebrospinal fluid was over, the flow was measured in drops per minute and total volume for 10 minutes. In this animal the first rapid escape was 8.2 cc. The slower flow is divided into three 10 minute periods: (a) before injecting extract and (b) (c) after injecting two separate quantities of the extract into the left femoral vein.

1st Period. Before Injection of Extract.

Drops per min.	Total in 10 min. cc.
3, 0, 0, 0, 1, 0, 3, 1, 1, 2	0.7
0, 1, 0, 0, 1, 0, 0, 0, 0, 1	0.2

2nd Period. 5 Cc. of Extract Injected into Left Femoral Vein.

Drops per min.	Total in 10 min. cc.
0, 5, 3, 2, 1, 1, 1, 1, 1, 1	1.2
1, 0, 0, 3, 3, 0, 0, 0, 1, 1	0.7
1, 0, 0, 1, 0, 1, 2, 1, 1, 1	0.5

3rd Period. 3.8 Cc. of Extract Injected into Left Femoral Vein.

Drops per min.	Total in 10 min. cc.
2, 1, 0, 1, 1, 0, 1, 1, 1, 1	0.4
1, 1, 0, 0, 1, 0, 0, 0, 1, 0	0.2
0, 0, 0, 0, 0, 0, 0, 0, 0, 1	0.05

The effect of the choroidal extract is observed after each injection, but not so markedly after the second injection.

The preliminary test on monkeys was even more satisfactory. The extract was injected into the basilio vein of a *Macacus rhesus* in an amount of 10 cc. in an animal weighing 3.5 kilos, without producing an observed ill effect. Two protocols of preliminary experiments on monkeys are given.

Oct. 22, 1917. Monkey A, *Macacus rhesus*; weight 5 kilos. Anesthesia: ether, morphine, and urethane. Puncture of subcerebellar cistern. The periods and readings are the same as in the previous protocol. The first flow of fluid following the puncture was 4.4 cc.

1st Period. Before Injection of Extract.

Drops per min.	Total in 10 min. cc.
2, 1, 1, 0, 1, 1, 1, 2, 5, 0	0.8
3, 2, 1, 1, 1, 1, 1, 0, 0, 0	0.5

2nd Period. 3.5 Cc. of Extract Injected into Basilic Vein.

Drops per min.	Total in 10 min. cc.
0, 0, 2, 2, 1, 1, 1, 1, 2, 2	0.9

Oct. 25, 1917. Monkey B, *Macacus rhesus*; weight 3.5 kilos. Anesthesia: ether, morphine, and urethane. The first flow of fluid following the puncture was 4.1 cc.

1st Period. Before Injection of Extract.

Drops per min.	Total in 10 min. cc.
1, 4,* 2, 1, 1, 2, 4,* 3, 1, 1	1.6
2, 0, 0, 0, 0, 0, 0, 0, 0, 0	0.1

2nd Period. 6 Cc. of Extract Injected into Basilic Vein.

Drops per min.	Total in 10 min. cc.
1, 1, 1, 1, 1, 1, 1, 1, 1, 1	0.7
1, 0, 0, 1, 1, 0, 1, 1, 1, 1	0.3

* In the interval represented by the two asterisks ether was administered.

The deduction from the three preliminary experiments is to the effect that the choroidal extract which we prepared and employed was an active one. There is another point which may be mentioned here. In order to obtain the stimulating effect of the extract, it

would appear that the animals need to be in good condition. Two monkeys in an advanced stage of tuberculosis showed no effect from the injections.

Experiments with Choroid Extract.

In carrying out experiments with the extract, the quality of the virus is of prime importance. It must be of such a degree of activity that it will not itself induce infection by simple intravenous injection, and yet it must be active enough to cause infection under conditions in which it is enabled to pass the choroidal-meningeal barrier. The importance of this consideration is illustrated by the first protocols.

Experiment 1.—Control A, *Macacus rhesus*. Dec. 5, 1917. Intravenous injection of 40 cc. of centrifugate of 5 per cent emulsion of fresh mixed virus. Dec. 11. Legs weak or paralyzed; right deltoid weak. Dec. 13. Both legs paralyzed; tremor of head. Dec. 14. Prostrate. Dec. 19. Died.

Autopsy.—Lesions of poliomyelitis.

Monkey C, *Macacus rhesus*. Dec. 5, 1917, 12.50 p.m. Intravenous injection of 40 cc. of centrifugate as in control. 12.55, 1.25, and 4.55 p.m. Intravenous injection of choroid extract. Dec. 6, 11 a.m. and 5 p.m. Injection of choroid extract. Dec. 12. Left facial paralysis and slight ataxia. Dec. 14. Legs paralyzed; deltoids weak. Dec. 17. Arms and back weak; lies down. Dec. 19. Improving. Jan. 2, 1918. Recovering use of limbs. Jan. 8. Recovered except for residual paralysis of legs.

Monkey D, *Macacus rhesus*. Dec. 5, 1917, 1.05 p.m. Intravenous injection of 40 cc. of centrifugate as in control. 1.10, 1.40, and 5.10 p.m. Injection of choroid extract. Dec. 6, 11 a.m. and 5.10 p.m. Injection of choroid extract. Dec. 7 and 8. Injection of choroid extract. Dec. 10. Right facial and double deltoid paralysis. Dec. 11. Died.

Autopsy.—Lesions of poliomyelitis.

This experiment is wholly inconclusive as to any promoting effects of the choroidal extract after an intravenous inoculation of the virus. Since the virus was of so high a degree of activity as to induce a fatal infection in the control monkey, the occurrence of paralysis in the other two animals was to be expected. Moreover, a comparison of Monkeys C and D suffices to dissipate any notion that the choroidal extract might have the effect of minimizing the action of the virus, since Monkey C partially recovered from the paralysis. Indeed, this experiment is a pertinent illustration of the factor of individuality

in affecting the outcome of an attack of poliomyelitis in the monkey, as well as in man. This factor of individuality appears even more emphatically in the next experiment.

Experiment 2.—Control B, *Macacus rhesus*. Oct. 29, 1917. Intracerebral inoculation of 1 cc. of 5 per cent emulsion of fresh spinal cord and medulla from paralyzed monkey. Nov. 2. Excited; tremor of head. Nov. 3. Tremor increased; ataxic. Nov. 6. Right facial paralysis; left arm weak. Nov. 8. All limbs paralyzed; moribund; etherized.

Autopsy.—Lesions of poliomyelitis.

Control C, *Macacus rhesus*. Oct. 29, 1917. 50 cc. of centrifugate of fresh emulsion of brain and cord, same as Control B, injected intravenously. Nov. 5. Double facial paralysis; ataxia; paralysis of right deltoid. The paralysis extended rapidly so that by evening the animal was prostrate and death occurred during the night.

Autopsy.—Lesions of poliomyelitis.

Monkey E, *Macacus rhesus*. Oct. 29, 1917, 12.50 p.m. Intravenous injection of 50 cc. of centrifugate, same as Control C. 1, 1.30, and 5 p.m. 5 cc. of choroidal extract injected. Oct. 30, 11 a.m. and 5 p.m. 5 cc. of choroidal extract injected. Oct. 31 and Nov. 1. 5 cc. of choroidal extract injected. Nov. 5 and 6. No extract injected. Nov. 7. Ataxia; limbs weak. Nov. 8. Right facial paralysis; deltoids paralyzed; legs weak. Nov. 10. Prostrate. Nov. 12. Moribund; etherized.

Autopsy.—Lesions of poliomyelitis.

Monkey F, *Macacus rhesus*. Treatment identical with that of Monkey E, except that no intravenous injections of choroidal extract were given after Oct. 30. This animal never showed any symptoms and was dismissed from observation on Nov. 19, at which time it was perfectly well.

The only deduction from this experiment is to the effect that the virus was sufficiently active to cause infection and paralysis in two of three monkeys into which it was injected intravenously while a third monkey was sufficiently insusceptible to resist its power of inducing infection. The choroidal extract probably played no essential part in the results.

In order to determine directly whether the choroidal extract exercised a restraining influence on the development of the infection the next experiment was performed.

Experiment 3.—Control D, *Macacus rhesus*. Dec. 4, 1917. Intraspinous injection of 2 cc. of sterile isotonic saline solution. Dec. 5. Intravenous injection of 40 cc. of centrifugate of fresh mixed virus. Dec. 10. Tremor; ptosis. Dec.

11. Ataxia; left deltoid and right leg weak. Dec. 12. All extremities paralyzed; etherized.

Autopsy.—Lesions of poliomyelitis.

Test: Monkey G, *Macacus rhesus*. Dec. 4, 1917, intraspinal injection of saline solution and Dec. 5, 12.05 p.m., intravenous injection of virus as in the control. 12.10, 12.40, and 4.10 p.m. 5 cc. of choroidal extract injected. Dec. 6. Repeated injection of extract at 10.10 a.m. and 4.10 p.m. Dec. 11. Left facial and right leg paralysis. Dec. 12. Both legs paralyzed; arms weak. Dec. 14. All extremities paralyzed. Progressive recovery followed. Animal regained use of arms. Jan. 18, 1918. Died of intercurrent infection.

Autopsy.—Healed lesions of poliomyelitis.

It is obvious that the course of the infection was practically identical in these two animals, and no inhibitory effect of the choroidal extract can be discerned.

Passing now to a virus which is incapable in the quantity employed of inciting infection from simple intravenous injection, we find that the injection of the choroidal extract does not change the results.

Experiment 4.—Control E, *Macacus rhesus*. Nov. 13, 1917. Intravenous injection of 25 cc. of centrifugate of active fresh virus (emulsion of medulla and spinal cord of paralyzed monkey). No symptoms developed and the animal was dismissed from observation on Dec. 17, at which time it was perfectly well.

Monkey H, *Macacus rhesus*. Nov. 13, 1917, 12.55 p.m. Intravenous injection of 25 cc. of virus as in the control. 1, 1.30, and 5 p.m. Injection of 5 cc. of choroidal extract. Nov. 14, 11 a.m. and 5 p.m. Injection of 5 cc. of choroidal extract. Nov. 15 and 16. Injection of 5 cc. of choroidal extract. No symptoms developed and the animal, in perfect health, was dismissed from attention on Nov. 30.

Experiment 5.—Control F, *Macacus rhesus*. Dec. 18, 1917. Intravenous injection of 32.5 cc. of centrifugate of active fresh virus (emulsion of medulla and spinal cord of paralyzed monkey). No symptoms developed.

Monkey I, *Macacus rhesus*. 12 m. Intravenous injection of 32.5 cc. of centrifugate as in the control. 12.05, 12.35, and 4.05 p.m. 5 cc. of choroidal extract injected. Dec. 19, 10.15 a.m. and 4.05 p.m. 5 cc. of choroidal extract injected. Dec. 20 and 21. 5 cc. of choroidal extract injected. No symptoms developed. The animal was dismissed from observation on Jan. 8, 1918, at which time it appeared perfectly well.

DISCUSSION AND SUMMARY.

The experiments recorded in this paper serve, in the first place, to confirm the experiments of Dixon and Halliburton on the stimulating effect of intravenous injections of extracts of choroid plexus in

the secretion of the cerebrospinal fluid, and extend their observations to monkeys.

They bring out also the variable effects of the virus of poliomyelitis, variations affected by the quality of the virus and also by the individual powers of resistance to infection possessed by individual monkeys. These factors of variation must be taken into account in performing and interpreting experiments on infection and particularly those on immunity and specific therapy in relation to poliomyelitis.

In general it may be said that experimental infection by way of the blood is not easy to produce in monkeys unless some contributing factor, such as the existence of a coincident aseptic meningitis, operates at the same time. And yet Experiments 1 and 2 show that when the strength of the virus is great the injection of relatively considerable quantities suffices to induce infection and paralysis, but not in all instances.

The chief outcome of the experiments has been to determine the fact that when the intravenous inoculation of the virus does not in itself suffice to induce infection and paralysis, the intravenous injection of extracts of the choroid plexus, which in themselves excite the secretory functions which preside over the formation of the cerebrospinal fluid, is powerless to modify this result. This fact would seem to be of interest and importance, since it has already been shown that very slight structural changes in the meningeal-choroidal complex suffice to make possible or certain infection under these circumstances. Apparently mere augmentation, from time to time, of the secretory functions of the choroid plexus, through intravenous injection of an extract of the choroid plexus and while the virus is still circulating, is insufficient to insure passage of the virus from the blood into the nervous tissues, upon which infection depends. Neither does the augmentation exercise a restraining influence on the development of infection otherwise capable of taking place.

THE AUTODIGESTION OF NORMAL SERUM THROUGH THE ACTION OF CERTAIN CHEMICAL AGENTS. I.

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INTRODUCTION.

In contrast with the so called defensive ferment, or "*Abwehrferment*" of Abderhalden, which has been recently much studied and discussed, comparatively little attention has been paid to the proteolytic ferment in normal serum, to which only occasional brief references can be found.

Abderhalden¹ has stated that he sometimes found a proteolytic ferment in the sera of guinea pigs and rabbits, which he held to have arisen through the introduction of foreign proteins, such as those due to the ingestion of plants, or to infectious diseases, especially coccidiosis. Stephan² reported that guinea pig serum shows an apparently polyvalent proteolytic power. Fuchs³ found that rabbits inoculated with serum gave a positive ninhydrin reaction with other kinds of substrates, and he explained this result by assuming that the sera of herbivorous animals contain a comparatively large amount of dialyzable substance. Michaelis and von Lagermarck⁴ obtained a positive Abderhalden reaction not only with pregnant serum but also with non-pregnant and even male serum, and they came to the conclusion that they could not confirm the existence of the specific ferment in Abderhalden's sense. Van Slyke, Vinograd-Villchur, and Losee⁵ also

¹ Abderhalden, E., *Abwehrferment. Das Auftreten blutfremder Substrate und Fermente im tierischen Organismus unter experimentellen, physiologischen und pathologischen Bedingungen*, Berlin, 4th edition, 1914, 53-54.

² Stephan, R., *Die Natur der sogenannten Abwehrfermente*, *Münch. med. Woch.*, 1914, lxi, 801.

³ Fuchs, A., *Tierexperimentelle Untersuchungen über die Organspezifität der proteolytischen Abwehrfermente (Abderhalden)*, *Münch. med. Woch.*, 1913, lx, 2230.

⁴ Michaelis, L., and von Lagermarck, L., *Die Abderhaldensche Schwangerschaftsdiagnose*, *Deutsch. med. Woch.*, 1914, xl, 316.

⁵ Van Slyke, D. D., Vinograd-Villchur, M., and Losee, J. R., *The Abderhalden reaction*, *J. Biol. Chem.*, 1915, xxiii, 377.

found proteolytic ferment in non-pregnant human serum by means of Van Slyke's method of amino nitrogen determination.

The existence, then, in normal human and animal serum, of a non-specific proteolytic ferment which digests certain proteins other than the serum has often been proved, but little investigation into the nature of this ferment has hitherto been made. The question of autodigestion of normal serum has received some attention from a few investigators, Delezenne and Pozerski⁶ having observed the autolysis of the serum under the influence of chloroform.

The present paper deals with a phenomenon of the autodigestion of normal serum brought about with certain chemical agents under various conditions.

Materials and Methods of Study.

Guinea pig serum was used chiefly in the present investigation, because it possesses advantage over other sera in its constancy and its richness in the ferment in question. Since, to secure uniformity of results, it was necessary to provide a sufficiently large quantity of serum for each series of experiments, with small animals a pool had to be made of many specimens from animals killed at the same time. When guinea pigs were used, the blood was withdrawn from the heart under general anesthesia by means of a sterile test-tube provided with a sharp cannula. The blood was collected in a sterile paraffined centrifuge tube, and upon coagulation it was centrifuged to separate the serum from the clot. By this method a clear serum, absolutely free from any trace of hemolysis, may be obtained. It is important to note that for the demonstration of autodigestion of normal serum through the intervention of certain chemical substances no specimen which contains hemoglobin should be employed, since, as will be shown later, the presence of hemoglobin and stroma, whether homologous or alien, leads to the appearance of digestive products and renders the issue of the self-digestion of the serum indecisive. The experiments were carried out with fresh active serum, although it was found that the activity of the serum is not perceptibly impaired by standing at a temperature of 6°C. for many days.

The amino substances normally contained in serum were previously removed by dialysis. The serum was placed in sterile celloidin sacs and was allowed to dialyze for 5 hours at room temperature in a sterile salt solution which renewed itself from a flow from another bottle placed above the level of the dialysis vessel. The celloidin sacs were preserved in sterile distilled water with a layer of toluene

⁶ Delezenne, C., and Pozerski, E., *Compt. rend. Soc. biol.*, 1903, lv, 327, cited by Jobling, J. W., and Petersen, W., *J. Exp. Med.*, 1914, xix, 460.

and before use were washed repeatedly with sterile salt solution. A layer of toluene protected the serum from bacterial interference during dialysis. The volume of the serum at the completion of dialysis was increased from one and a half times to twice its original volume. To secure a constant concentration the dialyzed serum was diluted with sterile salt solution until the volume became twice that of the original serum; that is, the dialyzed serum was made one-half of the original concentration. The dialyzed serum thus obtained, when kept in the refrigerator at 6°C., does not lose its proteolytic power for a long time, at least not for 3 or 4 weeks. We therefore kept in this way a sufficient supply of serum to complete many successive experiments with the same material.

The technique for dialysis was somewhat similar to that recommended by Abderhalden. The dialyzing thimbles used were those made by Schleicher and Schüll bearing the mark of 579A. In order to select perfect thimbles, it was necessary to test beforehand their permeability and intactness by means of solutions of silk peptone (Höchst) and egg white. Those which leaked or showed unusual porosity or retardation of dialysis were discarded as unsuitable. A serum to be tested for digestion was measured into a thimble which stood inside a sterile Jena glass wide mouthed flask. The height of the thimble and that of the flask were about the same, and the former was held upright by the edge of the latter. 15 cc. of sterile distilled water were poured into the flask outside the thimble. At the termination of dialysis, the fluid outside the thimble, representing the dialysate, was removed for determination of the amount of dialyzable proteins diffused out of the serum contained within the thimble. For this purpose the ninhydrin reaction was resorted to.⁷ Since this reaction requires a temperature above 100°C. maintained for at least 1 minute, it was not easy to obtain a uniform and constant result, owing to rapid evaporation and frequent loss of the fluid incidental to the violent bubbling caused by the application of a direct flame to the test-tube containing the dialysate and ninhydrin solution. A few previous workers have attempted to eliminate errors arising from this source by using a liquid paraffin bath instead of a direct flame. The overboiling of the fluid from the test-tube placed in the paraffin oil bath at a temperature above 100°C. was greatly reduced by a specially devised stopper,⁸ but we have found this device of little value, since it fails to prevent the loss of fluid by explosive escape of vapor, which forces out the fluid gathering about the narrow exit for stream.

After experiments with various devices a satisfactory result was obtained with the use of one suggested by Dr. Noguchi and illustrated in Text-fig. 1. With this

⁷ The Van Slyke apparatus for the determination of amino nitrogen was also used in certain series of experiments where the amounts of the split products were sufficiently large to use this apparatus, but in ordinary experiments the amounts were too minute to permit its use.

⁸ Oeller, H., and Stephan, R., *Technische Neuerungen zur Dialysiermethode, Deutsch. med. Woch.*, 1913, xxxix, 2505.

apparatus only occasionally does a small amount of the fluid escape. It consists of a hard glass test-tube (Pyrex), 1 cm. in diameter and 20 cm. in height (*a*), connected through a perforated rubber stopper (*b*) with another, somewhat narrower test-tube (*c*), the mouth of which is drawn into a long narrow neck to fit the stopper, and which has a narrow side arm near the bottom. When connected, the smaller test-tube, with narrow openings at both ends, stands in-



TEXT-FIG. 1. Apparatus used for the ninhydrin test. *a*, test-tube connected through a perforated rubber stopper (*b*) with another, narrower test-tube (*c*). In the lower test tube is a glass bead (*d*) which facilitates uniform diffusion of heat during the boiling of the mixture of dialysate and ninhydrin solution (*e*).

verted. The stopper may be lifted out with the upper tube attached and the mixture (*e*) of the dialysate (5 cc. as a rule) and ninhydrin solution (1 cc. of a 1:1,000 solution for 5 cc. of dialysate) placed in the lower test-tube, with a glass bead (*d*), which facilitates uniform diffusion of heat during the boiling. The upper portion is then tightly refitted, and the fluid is ready for boiling. The paraffin oil bath is made by filling an enameled pan with a sufficient amount of

the oil to give a depth of about 12 cm., which will cover nearly two-thirds of the height of the lower test-tube containing the fluid for heating. The bath should have width enough to hold a metal rack containing several tubes, as it is a great advantage to heat the entire series of tubes used in the experiment at the same time. It may be mentioned that the heating period is an important factor in relation to the intensity of the ninhydrin reaction. The color which manifests itself on cooling is gradually increased as the heating period is prolonged, although it was impossible to ascertain definitely, on account of the rapid evaporation of the fluid, at what rate and how long the increase proceeded. It was found, however, that the reaction at the end of 1 minute was much weaker than that of 5 minutes, and that after 10 minutes much stronger than that of 5 minutes' duration. A comparison of the intensity of the reaction was, of course, made after the volume of the fluid had been restored to the original standard by adding distilled water to the 5 cc. mark in the tube. It is easily seen, therefore, that a reaction which increases in intensity through minute errors due to inaccurate time limits would be greater during the preliminary few minutes than at the end of 5 minutes or longer. For this reason, throughout the entire experiment, instead of the 1 minute period of other investigators, we heated the fluid for exactly 5 minutes at a temperature of $150^{\circ}\text{C}.$, or as near $150^{\circ}\text{C}.$ as possible, the temperature being maintained by means of an oil bath in a wind-proof hood. At the end of 5 minutes the tubes were taken out of the bath and left at room temperature for 30 minutes before the reaction was read. In order to obtain a uniform and comparable result the content of each tube, which was reduced almost one-half through evaporation, was filled with distilled water up to the original volume of the dialysate; namely, 5 cc. in our experiment. The intensity of the reaction varied from a mere nuance to a distinct violet, with many intermediate grades. It was therefore necessary to prepare a standard by which different degrees of the reaction could be determined. Alanine was selected for producing the required color reaction by ninhydrin. 0.01 cc. of this substance, in 0.1 N solution, gives a distinct violet color, while 0.0025 cc. gives only a faint violet, when present in 5 cc. of distilled water. It was therefore possible to prepare a series of tubes in which color scales, based upon the gradually increasing amounts of 0.1 N alanine solution, were obtainable.

In the present study the reaction produced by 0.01 cc. of a 0.1 N solution of alanine in 5 cc. of distilled water was chosen as the standard. In an estimation of color intensity there may be two procedures. One is to have many grades of the color for comparison with a given reaction. The other, which is the one adopted in the present work, is to have one standard and to estimate the intensity of a given reaction by noting the amount of distilled water necessary to reduce the color exactly to correspond with the standard. If a given reaction requires a quadruple dilution to reach the standard, its intensity must be considered quadruple the standard; a reaction requiring triple or double dilution would be triple or double in strength.

For practical purposes, we have arbitrarily designated the reaction ++++ when the standard was attained by diluting with 3 to 3.9 cc. of water, +++ with 2 to 2.9 cc., ++ with 1 to 1.9 cc., and + with 0.9 cc. or less. Reactions weaker than this were recorded as <+ and \pm , which corresponded with a mixture of 1 cc. of the standard and water up to 1 cc. and that containing more than 1 cc. of water, respectively. The reactions may be briefly summarized as follows:
++++ for a reaction requiring 3 to 3.9 cc. of water to make it correspond with the standard.

+++ for a reaction requiring 2 to 2.9 cc. of water.

++ for a reaction requiring 1 to 1.9 cc. of water.

+ for a reaction requiring 0 to 0.9 cc. of water.

<+ for a reaction corresponding with standard 1 cc. + water up to 1 cc.

\pm for a reaction corresponding with standard 1 cc. + water more than 1 cc.

The ninhydrin reaction with amino-acid undergoes, within a day or so, a rapid discoloration, which cannot be prevented even by preserving the tubes in a refrigerator at 6°C. A suitable substitute was sought, therefore, among various violaceous aniline dyes, and it was found that a certain high dilution of crystal violet resembles very much the ninhydrin reaction, when carefully adjusted to the standard color of the latter, and remains unaltered for a long time, provided it is kept in a dark refrigerator. The standard color solution of crystal violet was utilized for the reading of the reaction because of its stability. It should be added, however, that when subjected to further dilution, the relative color values and effect no longer run parallel.

The proteolytic activity of the serum was tested not only for the autodigestion caused by chemical reagents, but also by using as substrates some pure preparations of plant or animal proteins and various animal tissues or blood corpuscles. When animal tissues were used, they were freed from blood, boiled, and emulsified exactly as in the procedure recommended by Abderhalden. All these substrates were dialyzed in a celloidin sac before use in order to remove any dialyzable protein substances which might be contained in some of the preparations.

Occurrence of the Proteolytic Ferment in Normal Guinea Pig Serum.

To 2 cc. of the dialyzed guinea pig serum various substrates, as shown in Table I, were added and digested in thimbles for 16 hours at 37°C. The control tests done with each substrate alone gave no color reaction, whereas those done with 2 cc. of dialyzed guinea pig serum gave a reaction of only \pm .

As will be seen from the table, the guinea pig serum, when incubated with some animal and plant proteins, produces dialyzable substances which show a positive ninhydrin test. Whether the serum in this case really digested the substrates, or whether the former was

TABLE I.

Effect of the Proteolytic Ferment of Normal Guinea Pig Serum on Different Substrates.

Substrate boiled.	Nin-hydrin test.	Substrate boiled.	Nin-hydrin test.	Substrate boiled.	Nin-hydrin test.
Guinea pig liver.	+++	Rabbit placenta.	+++	Cat serum.	±
“ “ corpuscles.	+++	“ serum.	±	“ fibrin.	±
“ “ placenta.	+++	“ fibrin.	±	Sheep corpuscles.	+++
Chicken liver.	+++	Dog corpuscles.	+++	“ fibrin.	±
“ corpuscles.	+++	“ serum.	±	Egg white.	±
“ serum.	±	“ fibrin.	±	Casein (Hammersten).	+++
Rabbit liver.	+++	Cat liver.	+++	Edestin (Merck).	+++
“ corpuscles.	+++	“ corpuscles.	+++	Ricin (Merck).	++
Guinea pig serum.	±	Dog liver.	+++	Sheep serum.	±

brought to autodigestion only by the influence of the substrates, is not shown by this experiment. The question will be discussed in more detail below. Among the substrates tested, the serum and fibrin of various animals and egg white remained indifferent to the proteolytic ferment of serum. The presence of such a polyvalent proteolytic ferment in normal serum is already known.

Autodigestion of Normal Serum through the Action of Certain Chemicals.

Quite distinct from the proteolytic phenomenon already described is the autodigestion of normal serum brought about through the intervention of non-nitrogenous chemicals such as acetone, alcohols, and chloroform. Table II gives the results obtained when these

TABLE II.

Autodigestion of Normal Serum as a Result of Treatment with Certain Chemical Reagents.

Test No.	Dialyzed guinea pig serum.	Chemical reagents.	Digested.	Ninhydrin test.
	cc.			
1	2.0	Acetone (Kahlbaum) 0.8 cc.	In thimble at 37°C. for 16 hrs.	+++
2	2.0	Methyl alcohol (Kahlbaum) 1.0 cc.		+++
3	2.0	Chloroform (Kahlbaum), shaken, 2.0 cc.		+++
4	2.0	Salt solution 1.0 cc.		±

chemicals were added to the dialyzed guinea pig serum, and the mixture was incubated at 37°C. for 16 hours. This phenomenon seems to suggest a sort of activation of the serum ferment by these chemicals.

The object of the following experiment was to determine the optimal concentration of acetone for a given volume of serum in order to cause autodigestion. The dialyzed guinea pig serum (2 cc.) was mixed in test-tubes with 1 cc. of acetone of various concentrations. After standing for 30 minutes at room temperature, the contents of each test-tube were transferred into a thimble and digested at 37°C. for 16 hours. The dialysates outside the thimbles were tested with ninhydrin (Table III).

TABLE III.
Optimal Concentration of Acetone to Activate Serum.

Test No.	Dialyzed guinea pig serum.	Salt solution.	Acetone.	Concentration of acetone mixture.	Ninhydrin test.
	cc.	cc.	cc.	per cent	
1	2.0	0	1.0	33 $\frac{1}{3}$	—
2	2.0	0.3	0.7	23 $\frac{1}{3}$	+++
3	2.0	0.5	0.5	16 $\frac{2}{3}$	+
4	2.0	0.7	0.3	10	±
5	2.0	0.8	0.2	6 $\frac{2}{3}$	±
6	2.0	0.9	0.1	3 $\frac{1}{3}$	±
7	2.0	1.0	0	0	±

As will be seen from the table the optimal concentration of acetone is very limited, and, according to repeated tests, it lies between 23 and 28.5 per cent. Either a lower or a higher concentration than this causes less effect, and no digestion takes place beyond a certain point. An amount of acetone which is sufficient to produce a strong turbidity or precipitation in the serum destroys the serum ferment at the same time, and there is no means of securing an active ferment by precipitating it from the serum with acetone. The same is true when the serum is precipitated with alcohol. The extreme lability of the serum ferment against acetone and alcohol presents a striking contrast to pepsin, trypsin, and other ferments, which, as is well known, withstand treatment with these reagents.

In autodigestion the serum no doubt plays the part of the ferment solution as well as of the substrate; hence, the more serum is used,

ceteris paribus, for digestion, the more split products are to be found in the dialysate. The relation of varying amounts of serum to digestion, under constant acetone concentration, was considered in the next experiment (Table IV). The result shows that under the same conditions of total liquid volume and acetone concentration the concentrated serum solution produces more dialyzable substances than the diluted one.

TABLE IV.

Relation between Various Amounts of Serum and the Degree of Autodigestion under Constant Acetone Concentration.

Test No.	Dialyzed guinea pig serum.	Salt solution.	Acetone.	Ninhydrin test.
	cc.	cc.	cc.	
1	2.0	0.2	0.8	+++
2	1.0	1.2	0.8	+
3	0.5	1.7	0.8	<+
4	0.25	1.95	0.8	±
5	0.1	2.1	0.8	—
6	0.05	2.15	0.8	—
7	2.0	1.0	0	±

Influence of Higher Temperature on the Serum Protease.

The test-tubes, each containing 2 cc. of the dialyzed guinea pig serum, were placed in the water bath regulated at 37°, 55°, and 60°C., respectively. After 30 minutes the tubes were taken out of the baths and allowed to cool at room temperature. An adequate amount of acetone or substrate (boiled chicken liver) was then added to the serum to permit detection of the presence of the ferment (Table V). The proteolytic ferment of serum, as the result shows, survives exposure to 55°C. for 30 minutes, but it is completely destroyed by heating at 60°C. for 30 minutes. The serum to which a suitable amount of acetone or a substrate had been added did not undergo autodigestion when placed in the incubator at 55°C. The optimal temperature for the action of this ferment seems to be 37°C.

TABLE V.

Resistance of the Serum Protease to Temperature.

Test No.	Dialyzed guinea pig serum.	Temperature applied.	Further treatment.	Ninhydrin test.
	cc.	°C.		
1	2.0	37	Acetone 0.8 cc.	+++
2	2.0		Substrate.	+++
3	2.0		Salt solution 0.8 cc.	=
4	2.0	55	Acetone 0.8 cc.	+++
5	2.0		Substrate.	+++
6	2.0		Salt solution 0.8 cc.	=
7	2.0	60	Acetone 0.8 cc.	=
8	2.0		Substrate.	=
9	2.0		Salt solution 0.8 cc.	=

Digestion Experiment in Test-Tubes without Simultaneous Dialysis.

In the preceding experiment autodigestion proceeded simultaneously with dialysis, since the serum and substrates or chemical activators were placed in a dialyzing thimble from the beginning. The question naturally arose whether or not the rate of digestion would be equally great if the mixture were put in a test-tube instead of a dialyzing thimble. There was reason to think that certain chemical activators such as acetone or alcohols would exert in test-tubes an injurious effect upon the serum ferment when added in proportions optimal for a dialyzing thimble, because in the latter a continuous reduction of the chemicals through osmosis must constitute a factor for yielding a maximum hydrolysis. In other words, the amounts of the reagents for digestion in thimbles would be too large for an optimal action of the ferment in test-tubes. This proved to be the case, as may be seen from the experiment recorded in Table VI.

Test 1 is a control test, showing the digesting power of the serum alone, without any treatment. Test 2 is another control, which demonstrates positive autodigestion caused by acetone. Test 3 shows that the serum loses its proteolytic power when mixed with acetone and kept at a temperature of 37°C. for 30 minutes. Test 4 shows that the acetonized serum which stands at room temperature for 30

minutes and then at 37°C. for 30 minutes is also inactivated. Test 5 shows that inactivation also takes place when the serum is acetoneized immediately after being taken out of the water bath. Test 6 shows that a previous incubation of the serum for 30 minutes at 37°C. has no injurious effect upon the ferment action if acetone is introduced after the serum has been sufficiently cooled by standing 30 minutes after the bath.

The foregoing experiments indicate that the quantity of acetone inducing an optimal digestion of the serum in a dialyzing thimble destroys the ferment in 30 minutes when the mixture is kept at 37°C.,

TABLE VI.

Effect of Acetone upon the Serum Protease in the Test-Tube at Different Temperatures.

Test No.	Dialyzed guinea pig serum 2 cc. in test-tube.					Transferred into thimble; digested at 37°C. for 16 hrs.
	At 37°C. in water bath.	At room temperature.	Acetone.	At room temperature.	At 37°C. in water bath.	Ninhydrin test.
	<i>min.</i>	<i>min.</i>	<i>cc.</i>	<i>min.</i>	<i>min.</i>	
1						±
2		30	0.8	30		+++
3			0.8		30	±
4			0.8	30	30	±
5	30		0.8	30		±
6	30	30	0.8	30		+++

while no injurious effect can be detected when it is kept at room temperature for half an hour.

That the use of the dialyzing thimble is an important factor in attenuating the destructive action of acetone upon the ferment through rapid exosmosis of the reagent is shown by the presence in the dialysate of some acetone soon after dialysis began. Prevention of exosmosis of acetone from the dialyzing thimble by the addition to the outside water of acetone in exactly the same proportion as that contained in the serum within the thimble results in total inactivation of the serum protease, as will be seen from the following experiment.

In Test 7, 2 cc. of dialyzed guinea pig serum were placed in a thimble with 0.8 cc. of acetone. Instead of the usual distilled water 15 cc., a mixture of dis-

tilled water 10.7 cc., and acetone 4.3 cc., was placed outside the thimble. The concentration of acetone was then equal on both sides of the thimble. As was expected, at the end of the usual incubation period, no digestion was found to have taken place.

Unlike acetone, chloroform and tissue substrates exert no injurious action upon the ferment, even when employed in excessive quantities; hence autodigestion by means of these substances can be carried out in test-tubes.

Removal of the Activating Reagents from the Mixture with Serum.

The phenomenon of autodigestion of serum through the intervention of certain reagents, belonging chiefly to the group of so called fat solvents, arouses interest as to the causes underlying this interaction. With acetone and the simpler alcohols it was noticed that a faint opalescence appears when the reagents are mixed with serum in the optimal proportion. Whether or not this slight physical change has any relation to autodigestion is not apparent. Moreover, in the case of chloroform, which is an excellent activator, no perceptible change, except the emulsification of the serum, takes place. One might assume that autodigestion is brought about by the extraction of fatty and lipoidal substances from the serum proteins, thus enabling the serum protease to act upon the delipolyzed proteins. But ether, benzene, toluene, or petroleum ether, in spite of their delipolyzing powers, have no activating property. At all events, it seemed important to ascertain what would happen if the chemicals once mixed with the serum were extracted from the mixture. As will be shown in the following experiments, it was found that serum once acetonized or treated with other suitable chemical activators in proper proportions remains autodigestive even after the activators are completely removed. The continued presence of the activating reagents in the serum is not necessary in order to induce autodigestion.

The chemical activators can be eliminated from the mixture with serum either by (1) evaporation, (2) dialysis, or (3) treatment with other indifferent substances which free the serum from the activating chemicals.

Evaporation Method.—Evaporation by means of vacuum is preferable, because it can be done at a lower temperature and with the least risk of bacterial contamination. A temperature above 15°C. should be avoided, since, in the mixture with activators, the activity of the ferment is highly sensitive to higher temperatures.

A mixture of serum with an adequate amount of a chemical activator is put into a large sterile Petri dish, the cover replaced, and the whole placed in a desiccator, which is then exhausted by means of vacuum. As soon as the pressure drops below a certain point, the

TABLE VII.

Effect of the Removal of Acetone from the Serum Mixture by Evaporation in Vacuo.

Test No.	Dialyzed guinea pig serum.	Acetone.	Further treatment.		Digested in thimble. Dialysate.	
					Test for acetone.	Ninhydrin test.
1	cc. 2.0	cc. 0	Controls. No further treatment.		—	±
2	2.0	0.8			++	+++
3	2.0	0.8	Acetone evaporated to the point when bubbling ceased. Volume restored to 2 cc.		+	+++
4	2.0	0.8		Acetone 0.8 cc.	++	+++
5	2.0	0.8	Acetone completely removed by desiccation <i>in vacuo</i> . Volume restored to 2 cc.		—	+++
6	2.0	0.8		Acetone 0.8 cc.	++	+++
7	2.0	0.8		Boiled.	—	—

contents of the dish begin to bubble. During the bubbling care must be taken to avoid loss of the liquid by overflow by regulating the speed of evaporation. The liquid ceases to bubble in a few minutes, as much of the activating reagents is already driven out of the mixture, but the odor reveals the presence of the small amount remaining. For complete removal of the reagents, evaporation must be continued until the contents of the dish are quite or nearly dried up. The residue obtained is then redissolved in sterile distilled water of a volume equal to that of the original dialyzed serum.

Acetone, chloroform, and methyl alcohol can be easily removed by this method, on account of their having a lower boiling point than the higher alcohols. But the higher series of alcohols, having a higher boiling point than that of distilled water, cannot be satisfactorily eliminated by this method.

TABLE VIII.

Effect of the Removal of Chloroform from the Serum Mixture by Evaporation in Vacuo.

Test No.	Dialyzed guinea pig serum.	Further treatment.			Digested in thimble. Ninhydrin test.
1	cc. 2.0	Control. No further treatment.			±
2	2.0	Shaken with chloroform repeatedly; stood at room temperature for 30 min.			+++
3	2.0		Chloroform completely evaporated. Volume restored to 2 cc.		±
4	2.0			Shaken again with chloroform.	+++
5	2.0	Shaken with chloroform repeatedly; stood at 6°C. for 30 min.			+++
6	2.0		Chloroform completely evaporated. Volume restored to 2 cc.		+++
7	2.0			Shaken again with chloroform.	+++
8	2.0			Boiled.	—

The behavior of the serum ferment after it has been freed from its activators by evaporation is shown in Tables VII and VIII. These tables show that acetone and chloroform can be completely removed from the mixture without any loss in the autodigestive activity of the serum, since it had already been activated by the reagents (Table VII, Test 5; Table VIII, Test 6). There is, however, a slight difference between the two reagents in their mode of action. The activating action of acetone is rather rapid, while that of chloro-

form is much slower (Table VIII, Test 3), requiring nearly 2 hours to insure an activation which will endure after the evaporation of the chemical.

Dialysis Method.—This method can be used only for the elimination of water-soluble substances, such as acetone and the lower alcohols. It is unavailable for chloroform and certain higher alcohols which are insoluble or less soluble in water. In order to utilize

TABLE IX.

Effect of the Removal of Acetone from the Serum Mixture by Evaporation and Dialysis.

Test No.	Dialyzed guinea pig serum.	Acetone.	Further treatment.		Digested in thimble. Dialysate.	
					Ninhydrin test.	Test for acetone.
1	cc. 2.0	cc. 0	Controls. No further treatment.		±	—
2	2.0	0.8			+++	++
3	2.0	0.8	Acetone partly evaporated immediately after having been mixed with serum.	Residual acetone removed by dialysis for 2 hrs.	+++	+
4	2.0	0.8			±	—
5	2.0	0.8			+++ Acetone 0.8 cc.	++
6	2.0	0.8	Acetone partly evaporated after standing with serum for 30 min. at room temperature.	Residual acetone removed by dialysis for 2 hrs.	+++	+
7	2.0	0.8			+++	—
8	2.0	0.8			+++ Acetone 0.8 cc.	++

celloidin membrane for dialysis it was necessary to remove, by a brief preliminary evaporation *in vacuo*, much of the reagent from the mixture, as the presence of acetone in such a concentration may affect the membrane. Complete removal of the reagent is then effected by dialysis in celloidin sacs for 2 hours in running salt solution. This combined method was used for the serum employed in Table IX.

As Test 4 shows, digestion cannot take place when the acetone is removed immediately after being mixed with the serum. If removal is begun after the mixture has already been allowed to stand at room temperature for 30 minutes, however, there is no difference in the ultimate outcome (Test 7). It is therefore advisable, in order to insure a thorough activation, to keep the mixture of serum and acetone at room temperature for at least 30 minutes before further treatment is started.

Extraction with Indifferent Fat Solvents.—As indifferent substances for the removal of chloroform or acetone from the serum by extraction, ether and petroleum ether were used, since they were found to possess neither an activating nor an injurious effect upon the serum

TABLE X.

Effect of Extraction by Means of Petroleum Ether of the Acetonized Serum.

Test No.	Kind of serum.	Further treatment.	Digested in thimble. Ninhydrin test.
1	Extracted serum 2 cc.	Alone.	+++
2		Acetone 0.8 cc.	+++
3		Emulsion of residuum 0.5 cc.	+++
4	Unextracted dialyzed guinea pig serum 2 cc. (controls).	Alone.	±
5		Acetone 0.8 cc.	+++
6		Emulsion of residuum 0.5 cc.	±

ferment. It was understood from the beginning that even by repeated and renewed extractions the acetone or alcohols cannot be completely exhausted from the serum admixtures. However, a point of interest in this mode of extraction lies in the fact that by it not only the added chemicals, but also the native fats and lipoids are removed, as is not the case in the evaporation or dialysis methods. Methyl alcohol is far less amenable to extraction from its mixture with serum, either by ether or by petroleum ether. An experiment in which this method was used follows.

4 cc. of acetone were mixed with 10 cc. of dialyzed guinea pig serum in a large centrifuge tube. After the mixture had been standing for 30 minutes at room temperature 10 cc. of petroleum ether were added to the liquid, which was then shaken energetically. The

emulsified liquid was centrifuged and the clear upper layer, consisting of petroleum ether and acetone, separated with a pipette. The extraction procedure was repeated five times and the extracted serum subsequently placed in a vacuum apparatus in order to remove the petroleum ether. The portions of petroleum ether containing fractions of acetone and representing several renewed extractions were reunited and evaporated *in vacuo*. The residue was emulsified in 1 cc. of salt solution (Table X).

As far as the experiment is concerned, the extraction of the acetone from the acetonized serum with petroleum ether makes no difference in the digesting process (Test 1). In other words, the absence of the substances of serum soluble in petroleum ether and acetone has no influence on the autodigestion of serum. It is interesting to note further that the addition of the lipoidal emulsion had neither an inhibitory action nor an accelerating influence upon the ferment activity of either the extracted (Test 3) or the unextracted (Test 6) serum. There was no antiferment in this fraction against the serum protease in question.⁹

Influence of Reactions upon the Serum Protease.

It is well known that the activity of a ferment is greatly influenced by the reaction of the medium in which it is found. In order to ascertain the optimal reaction for the serum protease, experiments were performed in which the digestion of the serum was carried out in various reactions. For this purpose amounts ranging from 0.01 to 1 cc. of a 0.1 N solution of hydrochloric acid or sodium hydroxide were added to a number of test-tubes, each containing a mixture of dialyzed guinea pig serum 2 cc., and acetone 1 cc. The total volumes of the mixtures were made uniformly 4 cc. by adding salt solution in the necessary amounts. The mixtures were allowed to stand at room temperature for 30 minutes and then were transferred

⁹ The inactivity of the lipid and fatty constituents of serum as an antiferment is attributed by Jobling and Petersen (*J. Exp. Med.*, 1914, xix, 549) to an imperfect dispersion after they are once extracted. By saponification they found them to be highly antienzymic. It seems open to discussion whether the antienzymic property of an unsaturated soap can explain the original antiferment of the serum.

to a corresponding number of dialyzing thimbles for incubation. The thimbles were placed in dialyzing flasks containing distilled water to which such quantities of acid or alkali were added as would make the reaction correspond exactly with the acidity or alkalinity of the contents of each thimble. The digestion was continued for 16 hours at 37°C. On account of the disturbing effect of acid or alkali upon the ninhydrin reaction, the acidity or alkalinity of the

TABLE XI.

Effect of Acid and Alkali on the Autodigestion of Serum.

Test No.	Dialyzed guinea pig serum.	Ace-tone.	Acid or alkali.	Salt solution.	Concentration of reaction in medium.*	Digested in thimble. Ninhydrin test.
	cc.	cc.	cc.	cc.		
			0.1 N hydrochloric acid.			
1	2.0	1.0	1.00	0.00	N/40 hydrochloric acid.	—
2	2.0	1.0	0.50	0.50	N/80 “ “	—
3	2.0	1.0	0.25	0.75	N/160 “ “	—
4	2.0	1.0	0.10	0.90	N/400 “ “	±
5	2.0	1.0	0.05	0.95	N/800 “ “	+++
6	2.0	1.0	0.01	0.99	N/4,000 “ “	+++
7	2.0	1.0	0	1.00	0	+++
			0.1 N sodium hydroxide.			
8	2.0	1.0	0.01	0.99	N/4,000 sodium hydroxide.	+++
9	2.0	1.0	0.05	0.95	N/800 “ “	++
10	2.0	1.0	0.10	0.90	N/400 “ “	—
11	2.0	1.0	0.25	0.75	N/160 “ “	—
12	2.0	1.0	0.50	0.50	N/80 “ “	—
13	2.0	1.0	1.00	0.00	N/40 “ “	—

* The figures under this heading give the resulting degrees of the reaction in the mixtures. The alkalinity of the serum itself after dialysis is weaker than $\frac{N}{1,000}$ sodium hydroxide and is therefore ignored in the calculation (Test 7).

dialysates was neutralized upon the completion of digestion (Table XI). A parallel series of experiments was carried out with alanine solution as controls.

As may be seen from Table XI, the serum protease is highly sensitive to the change in the reaction of the medium. The optimal re-

action for the ferment action is that of the dialyzed serum, or at least is within the narrow limits on each side of it, either toward acid or alkaline. Even a slight deviation in the reaction beyond these limits affects the activity of the serum ferment.

Certain Chemical Reagents as Activators of the Serum Protease.

In addition to acetone, chloroform and some alcohols were found to be ferment activators, and there may be others which behave similarly. On the other hand, ethyl ether, petroleum ether, benzene, and toluene have neither an activating nor a paralyzing action. They are indifferent towards the serum protease.

Chloroform as a ferment activator has been much discussed in preceding sections. Chloroform has as much activating power as acetone. However, the simple addition of chloroform to serum does not have much effect. The mixture must be energetically and repeatedly shaken in order to insure activation. For digestion, the emulsion of the mixture as a whole may be placed in the incubator; or one may use only the upper semitransparent layer which appears when the emulsion is allowed to stand for a few minutes at room temperature, while the greater part of the clear transparent chloroform settles at the bottom of the tube. With chloroform there is no optimal proportion to be added to the serum; the ferment is not affected at all, even when the chloroform is added in excess to the serum. That chloroform requires a longer time for activating the serum ferment than does acetone has already been noted (Table VIII).

In the following experiments some monovalent saturated alcohols and ketones were tested for their activating property.

Varying amounts of different ketones and alcohols were added to 2 cc. of the dialyzed guinea pig serum in test-tubes. Before the addition of the reagents adequate amounts of salt solution were added to the serum in order that the total volume in each test should be 3 cc. With substances which are less soluble or insoluble in serum, the mixtures were repeatedly shaken. All the tubes were allowed to stand for 30 minutes at room temperature and then were transferred into dialyzing thimbles to be placed in the incubator at 37°C. for 16 hours (Tables XII and XIII).

The ketones and alcohols behave similarly towards the serum ferment. A certain optimal concentration activates ferment, and an excess injures it. Moreover, it seems to be a rule among the reagents that the higher molecular substances of the series are generally more active than the lower ones. The optimal concentration, therefore, for activating ferment was found to be approximately 33 per cent for methyl alcohol, 23 to 27 per cent for ethyl alcohol, and 20 per cent for isopropyl alcohol. This rule seems to apply also to

TABLE XII.

Activating Power of Ketones on the Serum Protease.

Dialyzed guinea pig serum.	Salt solution.	Reagent.		Test No.	Acetone.		Test No.	Methylethyl ketone.	
		Amount.	Concen- tration.		Appearance of mixture.	Ninhy- drin test.		Appearance of mixture.	Ninhy- drin test.
cc.	cc.	cc.	per cent						
2.0	0	1.0	33 $\frac{1}{3}$	1	Turbid.	—	7	Emulsified.	—
2.0	0.3	0.7	23 $\frac{1}{3}$	2	Slight tur- bidity.	+++	8	"	—
2.0	0.5	0.5	16 $\frac{2}{3}$	3	Clear.	+	9	"	+
2.0	0.7	0.3	10	4	"	=	10	Slight tur- bidity.	+
2.0	0.8	0.2	6 $\frac{2}{3}$	5	"	=	11	Clear.	=
2.0	0.9	0.1	3 $\frac{1}{3}$	6	"	=	12	"	=

* The optimal concentration of methylethyl ketone for digestion +++ lies between 10 and 16.7 per cent.

ketones, though the tested substances were very few. The rule is well defined only for the lower series of substances which can be mixed with water in any proportion. In the case of the higher series, which are less soluble in water, the relation is not so constant, as will be seen from the results of tests with butyl and amyl alcohols.¹⁰ Finally, the still higher series, such as octyl alcohols, which are not soluble in water, have no activating power for the serum ferment.

¹⁰ The isobutyl alcohol is soluble in 10.5 parts of water at 18°C., and the isoamyl alcohol in 39 parts of water at 16.5°C.

TABLE XIII.

Activating Power of Alcohols on the Serum Protease.

Reagent.		Methyl alcohol.		Ethyl alcohol.		Isopropyl alcohol.		Isobutyl alcohol.	
Amount.	Concentration.	Appearance of mixture.	Ninhydrin test.	Appearance of mixture.	Ninhydrin test.	Appearance of mixture.	Ninhydrin test.	Appearance of mixture.	Ninhydrin test.
cc.	per cent								
1.0	33 $\frac{1}{3}$	Opalescent.	+++*	Strong turbidity.	—	Strong turbidity.	—	Emulsified.	—
0.7	23 $\frac{1}{3}$	"	+	Opalescent.	+++	Opalescent.	+	"	—
0.5	16 $\frac{2}{3}$	"	<+	"	+	"	+++†	"	—
0.3	10	Clear.	±	Clear.	±	Clear.	±	Strong turbidity.	<+
0.2	6 $\frac{2}{3}$	"	±	"	±	"	±	Slight turbidity.	+++
0.1	3 $\frac{1}{3}$	"	±	"	±	"	±	Clear.	±
0.07	2 $\frac{1}{3}$							"	±
0.05	1 $\frac{2}{3}$							"	±

Reagent.		Isoamyl alcohol.		Amyl alcohol, active		Octyl alcohol, normal.		Octyl Alcohol 2.	
Amount.	Concentration.	Appearance of mixture.	Ninhydrin test.	Appearance of mixture.	Ninhydrin test.	Appearance of mixture.	Ninhydrin test.	Appearance of mixture.	Ninhydrin test.
cc.	per cent								
1.0	33 $\frac{1}{3}$	Emulsified.	<+	Emulsified.	<+	Emulsified.	±	Emulsified.	±
0.7	23 $\frac{1}{3}$	"	+	"	+	"	±	"	±
0.5	16 $\frac{2}{3}$	"	+	"	+++	"	±	"	±
0.3	10	"	+++	"	+++	"	±	"	±
0.2	6 $\frac{2}{3}$	"	+++	"	+++	"	±	"	±
0.1	3 $\frac{1}{3}$	Moderate turbidity.	+++	Slight turbidity.	+++	"	±	"	±
0.07	2 $\frac{1}{3}$	Slight turbidity.	±	Clear.	+	"	±	"	±
0.05	1 $\frac{2}{3}$	Clear.	±	"	±	"	±	"	±
0.03	1	"	±	"	±	"	±	"	±
0.02	$\frac{2}{3}$	"	±	"	±	"	±	"	±
0.01	$\frac{1}{3}$	"	±	"	±	"	±	"	±

* The methyl alcohol, when added in a 1.5 cc. amount to 2 cc. of dialyzed serum, destroys the ferment.

† The concentration of isopropyl alcohol giving the maximal digestion is about 20 per cent.

SUMMARY AND CONCLUSIONS.

1. By means of certain chemical reagents, normal guinea pig serum can be brought to autodigestion without the presence of any foreign substrate. There exists in normal sera a highly characteristic protease.

2. The serum ferment survives heating at 55°C. for 30 minutes, but is completely inactivated at 60°C. for the same length of time.

3. The autodigestion of serum requires a temperature of about 37°C., and no noticeable digestion takes place at a temperature of 16°C. or lower.

4. Autodigestion of the serum may be brought about by chloroform and various saturated monovalent ketones and alcohols of the lower series.

5. The ketones and alcohols have a certain narrow limit of concentration for activating serum, beyond which the ferment is destroyed, even at room temperature.

6. The ketones and alcohols in concentrations regulated to activate serum at room temperature destroy the ferment when allowed to action serum at 37°C. for 30 minutes. The elimination of the concentrated reagents from serum by evaporation or dialysis protects the ferment from their destructive action.

7. A certain length of time is required for the chemical activators to complete their action. In this respect chloroform is much slower than acetone.

8. The chemical activators may be removed from the activated serum by means of vacuum, dialysis, or extraction with certain indifferent chemicals without causing a return of the serum to its original non-autolytic state. Once activated by these reagents, the serum remains in the activated state, in spite of the removal of the activators.

9. The ferment is highly sensitive to the reaction of the medium, being readily inactivated when the reaction exceeds a certain narrow limit towards acid or alkaline. The optimal digestion is obtained with a faintly alkaline or neutral reaction.

This work was done in the laboratory of Dr. Hideyo Noguchi, under his direction.

THE AUTODIGESTION OF NORMAL SERUM THROUGH THE ACTION OF CERTAIN CHEMICAL AGENTS. II.

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It has been shown that normal serum contains a characteristic protease whose activity is revealed through the action of certain chemical activators.¹ The seroprotease shows a thermal resistance similar to that of certain proteolytic enzymes, but is peculiar in its ready destruction by the action of acetone or alcohol, to which other ferments manifest a high degree of resistance.

In the present paper we have considered the relation which this ferment bears to the various fatty and lipoidal substances and also the existence of an antiferment in serum and its relation to the seroprotease. The material and method of study have been described in the previous paper.¹

Relation of Neutral Fats, Fatty Acids, and Lipoids to the Serum Protease.

Since all the reagents, the activating effects of which have been discussed in the previous paper, belong to the group of so called fat solvents, it is not out of place to consider what part the fat or lipid bodies may play in the autodigestion of serum caused by these reagents. The experiments were carried out partly by adding excessive amounts of fats or lipoids to the digesting mixtures, and partly by removing the native fats and lipoids from the serum by the use of fat solvents.

Several preparations of neutral fats and lipid bodies were dissolved in acetone in high concentration, with the exception of lecithin, which, on account of its insolubility in acetone, was dissolved in methyl alcohol. Each substance was added to 2 cc. of the dialyzed

¹ Yamakawa, S., The autodigestion of normal serum through the action of certain chemical agents. I, *J. Exp. Med.*, 1918, xxvii, 689.

guinea pig serum in two different concentrations. Some of the solutions precipitated particles of the substance when mixed with the serum and formed a layer near the surface. After standing for 30 minutes at room temperature, the contents of each test-tube were transferred into a dialyzing thimble and incubated at 37°C. for 16 hours (Table I).

That cholesterol, lecithin, and the neutral fats such as triolein and tripalmitin, even when they are added in excess to the serum, are indifferent to the process of autodigestion is proved by these experiments. The weakness of digestion in cases where fatty acids are added to the serum may be explained in various ways. As was stated in the previous paper,¹ the serum ferment is extremely sensitive to an acid reaction and is undoubtedly influenced by the fatty acids. The inhibiting power of the oleic acid was found to be much stronger than that of the palmitic acid (Tests 13, 14, 17, and 18), when they are allowed to act upon the serum ferment in equal concentration. It is not improbable that the weakness of the latter is chiefly due to its inferior solubility in a medium containing much water and to its higher melting point.

The phenomenon might be explained in another way; namely, by a specific inhibiting power of an unsaturated fatty acid such as oleic against the serum ferment. Jobling and Petersen² found that the unsaturated fatty acids in serum act as antitrypsin, and that they can be removed by extraction with ether or chloroform. But their results with trypsin do not find an analogy with the serum protease. As has been said, acetone or chloroform can impart their activating power to the serum ferment, and they do so without eliminating any of the native elements from it; a subsequent removal of the reagents from the activated serum does not restore the original resistance to autodigestion. Moreover, ether, toluene, benzene, and petroleum ether do not act as activators for the serum protease. The following experiment was undertaken in order to determine the effect of complete removal of the fats, fatty acids, and lipoids from the serum upon the phenomenon of autodigestion.

² Jobling, J. W., and Petersen, W., The nature of serum antitrypsin, *J. Exp. Med.*, 1914, xix, 459.

TABLE I.

Effect of Fatty Substances on the Autodigestion of Serum.

Test No.	Acetone solution of fats added to 2 cc. of dialyzed guinea pig serum.		Acetone.	Acid reaction.		Ninhydrin test.
				In thimble.	Of dialysate.	
1	10 per cent oleic acid.	cc. 0.8	cc. 0	+	—	<+
2		0.08	0.72	+	—	++
3	10 per cent triolein.	0.8	0	—	—	+++
4		0.08	0.72	—	—	+++
5	0.3 per cent palmitic acid.	0.8	0	<+	—	++
6		0.08	0.72	<+	—	+++
7	0.3 per cent tripalmitin.	0.8	0	—	—	+++
8		0.08	0.72	—	—	+++
9	Cholesterol saturated.	0.8	0	—	—	+++
10		0.08	0.72	—	—	+++
11	Guinea pig serum alone (controls).		0.8			+++
12			0			±

Test No.	Chloroform solution of fats added to 2 cc. of dialyzed guinea pig serum.		Chloroform.	Acid reaction.		Ninhydrin test.
				In thimble.	Of dialysate.	
13	10 per cent oleic acid.	cc. 1.0	cc. 0	+	—	<+
14		0.1	0.9	+	—	++
15	10 per cent triolein.	1.0	0	—	—	+++
16		0.1	0.9	—	—	+++
17	10 per cent palmitic acid.	1.0	0	+	—	++
18		0.1	0.9	+	—	++
19	10 per cent tripalmitin.	1.0	0	—	—	+++
20		0.1	0.9	—	—	+++
21	Guinea pig serum alone (controls).		1.0			+++
22			0			±

TABLE I—*Concluded.*

Test No.	Dialyzed guinea pig serum.	1 per cent ovo-lecithin emulsion in salt solution.	Salt solution.	Methyl alcohol.	Acid reaction.		Ninhydrin test.
					In thimble.	Of dialysate.	
	cc.	cc.	cc.	cc.			
23	2.0	1.0	0	1.5	—	—	+++
24	2.0	0.1	0.9	1.5	—	—	+++
25	2.0	1.0	0	0	—	—	±
26	2.0	0	1.0	1.5	—	—	+++

10 cc. of guinea pig serum were completely dried in the desiccator by means of vacuum. The residue was ground into powder, placed in a flask, and treated with 100 cc. of absolute ether. The flask was kept for 48 hours in the refrigerator, repeatedly shaken at intervals, and the solvent three times renewed. At the expiration of this period the ether was decanted, the precipitate was washed with another 100 cc. of ether, and the trace of the solvent was removed *in vacuo*. The dried powder was then dissolved in 10 cc. of sterile distilled water and dialyzed in salt solution to remove the dialyzable substances. After dialysis the serum was diluted to 20 cc. with salt solution and used for the tests (Table II).

TABLE II.

Autodigestion of the Serum Delipolyzed with Ether.

Test No.	Extracted guinea pig serum.	Further treatment.	Ninhydrin test.
	cc.		
1	2.0	No further treatment.	±
2	2.0	Acetone 0.8 cc. added.	+++
3	2.0	Shaken with 1 cc. of chloroform.	+++
4	2.0	Methyl alcohol 1 cc. added.	+++
5	2.0	Substrate (chicken liver) added.	+++

The experiment shows that the extraction of fatty substances from the dried serum with ether causes no change with regard to the phenomenon of autodigestion of the serum.

The Inhibitory Substance in Native Serum against the Serum Protease.

It is generally known that human or animal serum has an inhibitory effect upon various proteolytic ferments, such as pepsin, trypsin, leukoprotease, and autolytic ferment. The results of the investiga-

tions on the influence of serum on the serum protease will be described here.

The investigation divided itself into two parts: (1) the digestion of heterologous substrate by the guinea pig serum ferment, and (2) the autodigestion of serum caused by the chemical reagents already mentioned. In the latter case particular care was taken to remove the chemical reagents completely from the treated serum before the sample of native serum which was to be tested for its inhibitory power was added, because, should any trace of the activators still be present, it would lead to an activation of the serum thus introduced. Acetone was used throughout the experiment because of the ease with which it can be completely removed from the serum mixtures. The dialyzed serum, acetonized and then deacetonized, will be designated, for the sake of brevity, as "activated serum."

The result shown in Table III indicates that the larger the amount of the dialyzed guinea pig serum added, the greater is the digestion of the substrate. On the other hand, the addition of a dialyzed horse serum caused neither increase nor decrease of digestion by guinea pig serum (Table IV). The horse serum itself was inactive.

The result of the autodigestion test with activated serum distinctly shows the presence of an inhibitory substance in a dialyzed but otherwise unmodified serum (Table V). The contradictory results in both cases will be discussed later.

The serum antienzymes directed against various proteolytic ferments disappear from the serum when the latter is heated to a certain temperature. The two following experiments were undertaken to determine the thermal resistance of the antiseroprotease.

1 cc. of dialyzed guinea pig serum was heated in the water bath at different temperatures for varying periods of time. The heated sera, after having been cooled, were added to 2 cc. of the activated serum in tubes, allowed to stand at room temperature for 30 minutes, and transferred as usual into thimbles for incubation and dialysis (Table VI).

According to this experiment, the inhibitory substance in unmodified or native serum withstands heating at 55°C. for 30 minutes, whereas it is destroyed by exposure at 60°C. for the same period. The thermal resistance of the antiseroprotease coincides with that

TABLE III.

Digestion of a Substrate with Guinea Pig Serum in Increasing Quantities.

Dialyzed guinea pig serum.	Salt solution.	Digested in thimble with chicken liver. Ninhydrin test.	Digested in thimble without substrate. Ninhydrin test.
cc.	cc.		
2.0	2.0	+++	±
3.0	1.0	++++	±
4.0	0	++++	<+

TABLE IV.

Effect of a Heterologous Serum on the Digestion of a Substrate by Guinea Pig Serum.

Dialyzed guinea pig serum.	Dialyzed horse serum.	Salt solution.	Digested in thimble with chicken liver. Ninhydrin test.	Digested in thimble without substrate. Ninhydrin test.
cc.	cc.	cc.		
2.0	0	2.0	+++	±
2.0	1.0	1.0	+++	±
2.0	2.0	0	+++	±
0	2.0	2.0	±	—

TABLE V.

Inhibitory Power of the Homologous Serum against the Autodigestion of an Activated Guinea Pig Serum.

Test No.	Activated guinea pig serum.*	Homologous guinea pig serum.*	Salt solution.	Digested in thimble. Ninhydrin test.
	cc.	cc.	cc.	
1	2.0	2.0	0	<+
2	2.0	1.0	1.0	<+
3	2.0	0.5	1.5	<+
4	2.0	0.25	1.75	++
5	2.0	0.1	1.9	+++
6	2.0	0.05	1.95	+++
7	2.0	0	2.0	+++
8	0	2.0	2.0	±

* Both sera were previously dialyzed, and the mixture of both had been allowed to stand for 30 minutes at room temperature before being placed in the incubator at 37° C.

TABLE VI.

Inactivation of the Antiseroprotease of Guinea Pig Serum by Heating.

Test No.	Activated guinea pig serum.	Guinea pig serum 1 cc. exposed to various temperatures.	Ninhydrin test.
	cc.		
1	2.0	Not heated. Clear.	<+
2	2.0	30 min. at 55°C. Clear.	<+
3	2.0	30 " " 60° " Slightly turbid.	+++
4	2.0	30 " " 65° " Opalescent.	+++
5	2.0	30 " " 70° " "	+++
6	2.0	5 " " 100° " Coagulated.	+++
7	2.0	Salt solution 1 cc.	+++
8	Salt solution 2 cc.	Not heated. Clear.	=

TABLE VII.

Thermal Resistance of the Protease and Its Antisubstance in Serum.

Tests for protease.				Tests for antisubstance.				Heated serum alone.	
Test No.	Native guinea pig serum 1 cc. heated at 55°C. for.	Acetone added.	Ninhydrin test.	Test No.	Native guinea pig serum 2 cc. heated at 55° C. for.	Activated guinea pig serum added.	Ninhydrin test.	Test No.	Native guinea pig serum 2 cc. heated at 55° C. for.
	min.	cc.			min.	cc.			min.
1	30	0.8	+++	6	30	2.0	<+	12	30
2	60	0.8	+	7	60	2.0	++	13	60
3	120	0.8	=	8	120	2.0	+++	14	120
4	240	0.8	—	9	240	2.0	+++	15	240
5	Guinea pig serum not heated, 2 cc.	0.8	+++	10	Guinea pig serum not heated, 1 cc.	2.0	<+	16	Guinea pig serum not heated, 2 cc.
				11	Salt solution 1 cc.	2.0	+++		

of the serum protease itself.³ This fact was proved again in the next experiment.

The unmodified or native guinea pig serum, 1 cc., was exposed to a temperature of 55°C. for various periods of time. Each heated serum was mixed

³ Yamakawa,¹ Table V.

with acetone or activated serum respectively, to be tested for its proteolytic and antiproteolytic power. After standing for 30 minutes at room temperature the mixtures were transferred into thimbles and placed in the incubator (Table VII).

Exposed to a temperature of 55°C., both the ferment and the antistubstance remain unimpaired for 30 minutes, but their activity gradually diminishes after a longer time, finally disappearing after 2 hours. A dissociation of the ferment from its antistubstance through heating was found to be impossible.

Effect of the Adsorbing Substances on the Serum.

Certain inorganic substances, which had been previously sterilized by heating, were put into the dialyzed guinea pig serum in a proportion of 5 gm. to 10 cc. The mixtures were allowed to stand at room

TABLE VIII.

Digesting Power of the Serum Treated with Adsorbents.

Test No.	Kind and amount of guinea pig serum.	Further treatment.	Ninhydrin test.
1	Serum treated with kaolin 2 cc.	No further treatment.	—
2		Acetone 0.8 cc. added.	—
3		Substrate added.	—
4	Serum treated with charcoal 2 cc.	No further treatment.	—
5		Acetone 0.8 cc. added.	±
6		Substrate added.	—
7	Serum treated with talc 2 cc.	No further treatment.	—
8		Acetone 0.8 cc. added.	±
9		Substrate added.	—
10	Serum treated with silicious marl 2 cc.	No further treatment.	—
11		Acetone 0.8 cc. added.	±
12		Substrate added.	—
13	Serum treated with barium sulfate 2 cc.	No further treatment.	—
14		Acetone 0.8 cc. added.	±
15		Substrate added.	—
16	Untreated serum 2 cc. (controls).	No further treatment.	±
17		Acetone 0.8 cc. added.	+++
18		Substrate added.	+++

temperature for an hour, with repeated shakings, and then centrifuged. The clear supernatant fluids were used for the experiment (Table VIII).

As may be seen from Tables VIII and IX, the proteolytic ferment can be easily removed from serum by adsorbents, but the antistubstance, on the other hand, still remains in the treated serum.

TABLE IX.

Antienzymic Action of the Serum Treated with Adsorbents.

Test No.	Kind and amount of guinea pig serum.		Activated serum added.	Acetone added.	Ninhydrin test.
		cc.	cc.	cc.	
1	Guinea pig serum treated with kaolin.	2.0	0	0	—
2		2.0	0	0.8	—
3		1.0	2.0	0	<+
4	Guinea pig serum treated with talc.	2.0	0	0	—
5		2.0	0	0.8	±
6		1.0	2.0	0	<+
7	Salt solution.	1.0	2.0	0	+++
8	Guinea pig serum.	1.0	2.0	0	<+

Occurrence of the Proteolytic Ferment and Its Antistubstance in the Sera of Different Animals.

It would surely have been an advantage if we could have found larger animals which would furnish us with a serum as rich in the

TABLE X.

The Proteolytic Ferment and Its Antistubstance in the Sera of Different Animals.

Kind of serum.	No. of tested specimens.	Dialyzed serum alone 2 cc.	Dialyzed serum 2 cc. + acetone 0.8 cc.	Dialyzed serum 1 cc. + activated guinea pig serum 2 cc.
Human serum.....	2	+	<+	<+
Dog ".....	5	±	<+	<+
Cat ".....	2	±	<+	<+
Rabbit ".....	8	±	<+	<+
Horse ".....	2	—	<+	<+
Guinea pig serum.....	Over 100	±	+++	<+

serum protease as that of the guinea pig. The results of examinations of various animal sera, however, showed that the guinea pig is the only animal whose serum is exceedingly rich in the proteolytic ferment. On the other hand, the sera of other animals, while poor in their content of protease, contain a considerable amount of the antistubstance capable of counteracting the action of the autolytic ferment of guinea pig serum. The result of the digestion tests with the sera of different animals is shown in Table X.

Mode of Digestive Action of the Serum Ferment.

It has been stated in a previous paragraph that the proteolytic ferment of serum, when it is incubated with substrate, can produce the dialyzable substances despite the presence of native serum, while in the autodigestion of activated serum, the ferment action is inhibited by the addition of native serum. There seems to be a certain difference in the mode of action in the two instances.

The explanation of the autodigestion of the activated serum may probably be sought in the destruction of paralysis of the antienzymic substance through the treatment. Reagents such as certain ketones and alcohols, when their optimal concentration for activation is reached, may destroy the antienzyme, but not the enzyme, thus enabling the latter to exert its full activity upon the serum proteins. The concentration of reagent which dissociates the ferment from its antistubstance lies between narrow limits, and when it exceeds the upper limit, the ferment itself is also destroyed.

In autodigestion the protein in the treated serum must serve as substrate, because there is nothing else present to be hydrolyzed. But what is the origin, then, of the dialyzable substance produced when the serum is incubated with various tissue substrates? There are two possibilities for the source of the protein derivatives: first, the substrates may be directly digested by the serum ferment; second, it may be assumed that the homologous tissues are not really digested, but that they act only as an adsorbing agent which removes the antienzyme and leaves the freed autolytic ferment to digest its own serum protein. The latter explanation was advanced by Bron-

fenbrenner⁴ in the Abderhalden reaction, in which pregnant human serum, when incubated with placenta tissue, gives a positive ninhydrin test. He states that pregnant serum is able to show auto-digestion in the incubator when allowed to remain in contact with

TABLE XI.

Antienzymic Action of Normal Serum after Treatment with Substrate at 0.5°C.

Test No.	Dialyzed guinea pig serum.	Further treatment.			Digested in thimble Ninhydrin test.
1	2.0	Substrate added. Tubes left at 0.5°C. for 16 hrs.	Centrifuged. Substrate removed. Supernatant fluid alone used for tests.		±
2	2.0			Boiled.	—
3	2.0			Acetone 0.8 cc.	+++
4	1.0			Activated guinea pig serum 2 cc.	<+
5	2.0		Substrate <i>in situ</i> .		+++

Control tests.

6	2.0	Without any treatment.	±
7	2.0	Acetone 0.8 cc. added.	+++
8	2.0	Substrate added.	+++
9	1.0	Activated guinea pig serum 2 cc. added.	<+
10	Salt solution 1 cc.	Activated guinea pig serum 2 cc. added.	+++

placenta tissue for 16 hours on ice and then separated from the substrate. He ascribes the phenomenon to adsorption of the anti-enzymic substance by the substrate impregnated with a specific antibody contained in the serum of a pregnant subject.

The next experiment was undertaken to determine whether this

⁴Bronfenbrenner, J., On the present status of the Abderhalden reaction, *J. Lab. and Clin. Med.*, 1915-16, i, 79.

mode of interpretation was applicable in our case, in which a heterologous, non-specific substrate is treated with normal serum (Table XI).

Tests 1 and 4 show that normal guinea pig serum, when it is kept on ice with substrate, is neither activated nor deprived of its antienzymic substance. In other words, the normal serum is indifferent to the treatment, contrary to the result which Bronfenbrenner reported to have obtained with human pregnant serum and placenta tissue. But this result does not exclude the possibility of the adsorption of the antienzyme by ordinary substrates in the incubator at a temperature of 37°C.

To determine the fate of the antienzymic substance in serum, after digestion, the serum was treated according to four methods, as follows:

Serum A.—8 cc. of the dialyzed guinea pig serum were kept in the incubator with substrate in four thimbles for 16 hours, 2 cc. being placed in each thimble. At the expiration of this time, when the dialysate showed a ninhydrin reaction of + + +, the serum in the thimbles was separated from the substrate layer and every trace of the latter removed by means of centrifugation. The serum was then dialyzed in a celloidin sac against salt solution to eliminate the split products of protein contained in it.

Serum B.—8 cc. of the activated guinea pig serum were kept in thimbles in the incubator for 16 hours. The ninhydrin test of the dialysate showed a reaction of + + +. The sera in the thimbles were reunited and dialyzed as mentioned above.

Serum C (Control 1).—The dialyzed guinea pig serum, 8 cc., without any substrate and without being activated, was treated in the same way as the other two sets of serum; *i.e.*, incubated in thimbles and afterwards dialyzed.

Serum D (Control 2).—The dialyzed guinea pig serum without any preliminary treatment.

These sera were further treated as shown in Table XII and digested in thimbles at 37°C. for 16 hours. The dialysates were tested as usual.

The result of this experiment indicates that the serum, the proteolytic power of which has already been exhausted by treatment with substrate, still contains its antienzymic substance (*Serum A*, Test 4), while the latter is no longer found in the activated serum after digestion (*Serum B*, Test 4). There is no doubt that in the former case the digestion can take place in spite of the presence of

the antienzymic substance. We have no more reason in this instance to assume the occurrence of an indirect digestion of serum protein, due to the absorption of the antienzymic substance through the substrate, because the substrate does not absorb the antienzyme under the experimental conditions here recorded. It seems justifiable, therefore, to conclude that the serum ferment directly digests the protein of the heterologous substrate, while in the case of the activated serum, the ferment splits its own serum protein after the antienzymic substance has been removed by the treatment with ketones or alcohols.

TABLE XII.

Fate of the Antienzyme Substance in Serum after Digestion.

Test No.	Amount of serum.*	Further treatment.	Serum A	Serum B	Serum C	Serum D
	cc.					
1	2.0	No further treatment.	—	—	—	±
2	2.0	Acetone 0.8 cc.	—	±	++	+++
3	2.0	Substrate.	—	+	++	+++
4	1.0	Activated guinea pig serum 2 cc.	<+	+++	<+	<+
5	1.0	Dialyzed guinea pig serum 2 cc. + substrate.	+++	+++	+++	+++

* The volume of the serum was increased after secondary dialysis by about one-fourth.

SUMMARY AND CONCLUSIONS.

1. The neutral fats, fatty acids, and lipoid bodies of serum seem to play no part in autodigestion. Neither the addition of fats or lipoids in excess to the serum, nor their removal by extraction with ether influences the phenomenon of autodigestion.

2. There is present in native serum an antienzymic substance which is closely related to the autolytic ferment of serum.

3. The antiseroprotease of normal serum has almost the same thermal resistance as the seroprotease; that is, it survives heating at 55°C. for 30 minutes but is completely inactivated at 60°C. for the same length of time.

4. The ferment can be removed from the serum by means of inorganic adsorbents, but the antienzymic substance remains in the treated serum.

5. The autolytic power of the sera of man and other animals is much weaker than that of guinea pig serum, but they contain as much as does the latter of the antistubstance which inhibits the digestion of the activated guinea pig serum.

6. The autodigestion of the activated serum is due to the splitting of the serum protein by the proteolytic ferment of the same serum and is brought about by the destruction of the antienzymic substance by the chemical reagents. On the other hand, the digestion products in a mixture of a foreign substrate and guinea pig serum are derived from the direct digestion of the substrate by the serum ferment. This digestion takes place in spite of the presence of the antiseroprotease. The serum separated from the substrate can no longer produce a split product, but is as actively antienzymic as the original serum and undergoes autodigestion only when treated with acetone or other chemical activators.

This work was done in the laboratory of Dr. Hideyo Noguchi, under his direction.

ANTIBODY PRODUCTION AFTER PARTIAL ADRENAL-ECTOMY IN GUINEA PIGS.

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Aside from the well established action of products of adrenal secretion upon sympathetic nerve endings and their consequent effect upon striated and smooth musculature, there has been assigned to the adrenal glands a detoxifying action upon endogenous and exogenous poisons which would class them among the specific defensive mechanisms of the body. Studies of this action have been limited largely to observations and experiments to show the effects of acute and chronic intoxications upon the adrenal glands, the symptomatology of adrenal hyperplasia or degeneration, the effect of partial or complete extirpation upon the toxicity of various substances, or the neutralization of toxins by adrenal extracts in the test-tube or the living animal. The literature is confused and contradictory, and much of the evidence brought forward is speculative and indirect, due in part to the use of the death or survival of the animal as sole indicator, and the consequent introduction of many unknown factors into the experimental equation, without critical analysis of the mechanism involved.

Only a few experiments have come to light which bear directly upon a possible relation between the adrenal glands and the recognized immunity factors of the defensive mechanism, and these reported findings are almost entirely of a negative significance. Thus Josué and Paillard¹ found that injections of adrenalin or of adrenal extracts into rabbits had no effect on the opsonic properties of the blood. Hektoen and Curtis² report that "Adrenalectomy in normal dogs, and in dogs at the height of the antibody curve after the injection of rat blood, did not cause any fall in the antibody content of the blood serum, as determined by hourly

¹ Josué, O., and Paillard, H., Influence de l'adrénalin sur le pouvoir opsonique (Première note), *Compt. rend. Soc. biol.*, 1910, lxxviii, 657.

² Hektoen, L., and Curtis, A. R., The effect on antibody production of the removal of various organs, *J. Infect. Dis.*, 1915, xvii, 409.

observations after the operation and until death." Gay and Rusk³ in presenting studies on antibody formation, in which the literature is reviewed, make no mention of the adrenals as possible sources of immune substances, and all the positive evidence reported in regard to the origin of immune bodies points to other organs, notably the lymph glands, bone marrow, and spleen, as the tissues probably concerned. A solitary communication by Cattoretti⁴ states that the addition of pancreas extract to the blood of adrenalectomized rats gave a marked lowering of the surface tension (miostagmin reaction) compared with the normal lowering produced by the extract.

There is no confirmed evidence that the adrenal glands play an active part in antibody formation, or in the known immunity reactions of defense against bacterial invasion. On the other hand, the few experiments which have been made to study such a relation have given negative results.

As a part of a more general investigation of the possible relation of glands of internal secretion to immunity processes we have made experiments on the effect of partial adrenalectomy upon antibody formation in guinea pigs. For this purpose the animals were subjected to operation before or after immunization with a typhoid vaccine or with washed red blood corpuscles of the hen, and their typhoid agglutinins or hemolysins and hemagglutinins titered at intervals during the course of antibody production.

Healthy adult guinea pigs, usually males, weighing from 300 to 400 gm., served as the experimental animal. For the purposes of the experiments it was necessary that the animals should survive during the interval required for subsequent active immunization or for the change in antibodies already in circulation, in the event that adrenalectomy modified the response. Consequently, complete removal of the glands was interdicted, since guinea pigs are practically without accessory adrenal tissue and almost invariably die within a few hours of a total extirpation.⁵

Operations for the partial removal of the adrenals were performed

³ Gay, F. P., and Rusk, G. Y., Studies on the locus of antibody formation. *Tr. XVth Internat. Cong. Hyg. and Demog., Washington (1912)*, 1913, ii, 328.

⁴ Cattoretti, F., Ueber die Meistagminreaktion bei den weissen Ratten nach Extirpation der beiden Nebennieren, *Wien. klin. Woch.*, 1911, xxiv, 637.

⁵ Lucien, M., and Parisot, J.-V.-J., Glandes surrénales et organes chromaffines, Paris, 1913, 108.

with careful aseptic technique, and the endeavor to reduce shock to the minimum by prompt hemostasis, the use of a warm pad during and after operation, machine-controlled anesthesia with constant ether concentration, and saline injections when indicated. We deemed it essential carefully to avoid complications due to infection or to injury to other organs in the course of the operation.

Operation.—Oblique incisions on both sides, separating the last two ribs, and extending to the edge of the sacrospinal muscles gave a clear exposure of the glands with the least disturbance of the abdominal contents. The adrenals were dissected free to the hilum, caught in a delicate curved mosquito clamp at the base or through their substance, and cut free distal to the clamp. Only slight bleeding resulted if the clamp was left in place a short time. The glands or portions of glands removed were weighed to estimate the amount taken and sectioned for comparison post mortem with the segment left in place.

It was soon found that the guinea pig could stand the loss of the whole of one gland and from one-half to three-fourths of the other. If too much tissue was taken the animal died in from a few hours to several days, after showing characteristic symptoms. A marked fall in temperature (to 28°C. in one instance), extreme prostration, gasping respiration, intermittent clonic convulsions, and, in males, the extrusion of semen immediately preceded death. These findings are in accord with former reports.

On the other hand, surviving guinea pigs recovered quickly from the operation and remained well for months, sometimes losing at first 50 to 100 gm. in weight, which was often recovered later. Some of the animals died during the course of the experiments, however, from hemopericardium after cardiac puncture or from an intercurrent lung epizootic prevalent among the stock.

Technique of the Serum Reactions.—The strain of *B. typhosus* chosen was a stock culture known as "Sen," recovered by Dr. Bull from an ampule of Besredka's sensitized vaccine. It had been on artificial media for several years and combined ready agglutinability with a high toxicity for guinea pigs, often killing them in the usual immunizing doses. For this reason and to obtain exact dosage, a vaccine was prepared by suspending 24 hour growths from Blake bottles in saline solution, killing the bacilli with chloroform, disrupting them by repeated freezing and thawing, dehydrating *in vacuo* over sulfuric acid, and powdering in a mortar. Weighed quantities were resuspended in normal saline solution, extracted by shaking for several hours, and measured doses corresponding to 0.5

to 2 mg. of the powder were injected intraperitoneally at 3 to 4 day intervals for three doses. Initial agglutinin titers ranged from 1:640 to 1:5,120 on the 7th to 10th day after the final injection. Ten of twenty-four guinea pigs gave an initial titer of 1:1,280. In order to simplify the experiments only the typhoid agglutinins were followed. Casual tests showed that this method of immunization, while simple and rapid, did not produce precipitins or complement-fixing bodies in concentrations suitable for investigation.

Other guinea pigs were immunized with red blood corpuscles of barred Plymouth Rock chickens. Following Coca's⁶ schedule three intraperitoneal doses of 0.5 to 1.5 cc. of washed corpuscles, made up to the original blood volume, were injected at 4 day intervals and the guinea pigs were first bled a week later. The first hemagglutinin titers ranged from 1:160 to 1:1,280, hemolysins appearing in dilutions five times as great, taking into account the dilutions involved in the hemolytic system. For the agglutination tests one drop of a suspension of *B. typhosus*, Sen, or of washed hen corpuscles (10 per cent of the original blood volume), was added to a 1 cc. volume of successive dilutions of the fresh, inactivated guinea pig serum. The tubes were read first after 2 hours at 37°C. and 2 hours at room temperature. A confirmatory reading was taken after the tubes had stood in the ice box over night. To test hemolysins, 0.25 cc. volumes of inactivated experimental serum, fresh guinea pig complement 1:10, and 5 per cent hen corpuscle suspension were made up with 0.5 cc. of saline solution, following the usual technique of the Wassermann reaction. These tubes were incubated 1½ hours, being shaken at the half hour and hour, and read immediately, and after standing in the ice box over night. All the tests were performed with the usual controls.

With these methods several series of experiments were performed. Usually three guinea pigs formed the experimental unit. In some instances two were partially adrenalectomized, the other serving as a normal control. In other cases two animals served as controls, one normal, the other after an operation similar to double adrenalectomy except for the removal of the glands. The control operated animals showed that the operation itself had no influence on antibody formation.

Series I.

In some of the experiments in this series the guinea pigs were first immunized with *Bacillus typhosus* vaccine and their agglutinin titers recorded on the 7th day after the third injection. Within a few days

⁶ Coca, A. F., A rapid and efficient method of producing hemolytic amboceptor against sheep corpuscles, *J. Infect. Dis.*, 1915, xvii, 361.

(8 to 11 days after immunization) the adrenalectomies or control operations were performed and the agglutinin titers followed in 2 cc. samples of blood obtained by cardiac puncture at intervals during the succeeding weeks and months. In other experiments the operations preceded immunization, which was begun from 1 to 52 days later. In a few instances one gland or a part of a gland was removed, the

TABLE I.

Differences in Agglutinin Titer of Various Sera on Reexamination after an Interval of Time.

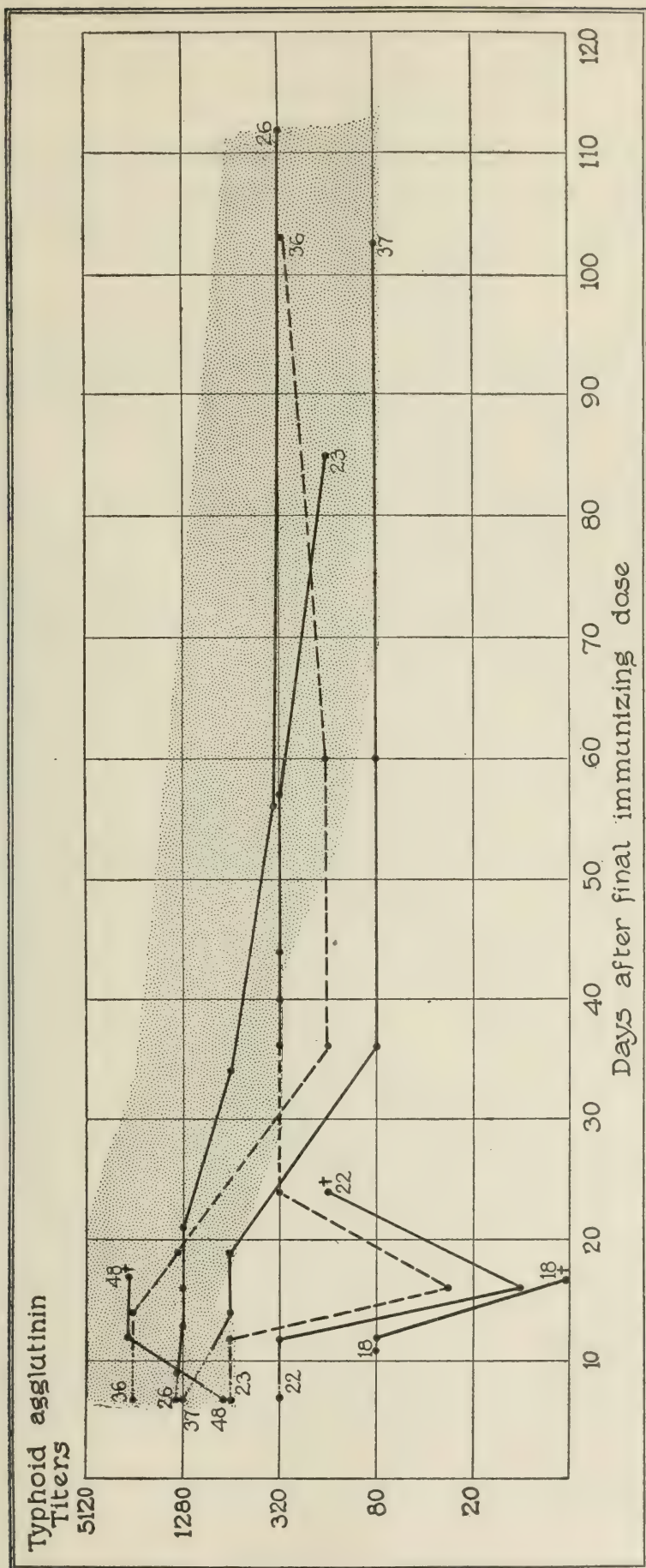
Animal No.	Condition.	Serum of.	Interval between tests.	First titer.	Second titer.
			days		
22	Double adrenalectomy almost complete.	Dec. 27, 1916	35	1:30	1:640
		Jan. 4, 1917	26	1:320	1:1,280
23	Right adrenal removed.	Dec. 27, 1916	35	1:160	1:640
		Jan. 4, 1917	26	1:320	1:320
	Three-fourths of left adrenal removed.	Jan. 16, 1917	15	1:320	1:640
		" 20, 1917	7	1:320	1:320
		Mar. 5, 1917	7	1:160	1:320
24	Normal.	Dec. 27, 1916	35	1:1,280	1:2,560
		Jan. 4, 1917	26	1:1,280	1:2,560
	Control operation.	Jan. 16, 1917	15	1:1,280	1:1,280
		" 20, 1917	7	1:640	1:1,280
		Mar. 5, 1917	7	1:160	1:320
2	Double adrenalectomy.	Mar. 5, 1917	7	1:160	1:640
		" 12, 1917	14	1:640	1:640
39	Normal.	Mar. 5, 1917	7	1:160	1:160

guinea pig immunized, and the second gland or part of it taken at a later date. These two sets of experiments, in which the effect of partial adrenalectomy was studied in animals previously or subsequently immunized to *Bacillus typhosus*, form a group in which all the control animals may serve as a basis for comparison with those on which the extirpation of adrenal tissue was performed. The agglutinin curves of the control animals showed considerable variations

due to individual reaction, so that a plot of all the normal curves gives a confusion of lines within rather wide limits on the chart. Obviously comparison of the antibody curve of an adrenalectomized animal with its own control alone might indicate differences in reaction not due essentially to the loss of adrenal tissue. Therefore it seems best simply to outline an area which covers all the variations in reaction found in normal guinea pigs. Against this normal area the separate curves of agglutinin formation in the experimental animals may be charted. Following this method Text-fig. 1 shows the curves of seven guinea pigs which were adrenalectomized subsequent to immunization. It will be seen that the titers of all but three of them fall practically within the limits of normal variation. The findings in the exceptional cases require special comment.

Immediately after operation the agglutinin titers of the sera of these animals appeared to drop sharply almost to zero, with as sharp a rise later in the two guinea pigs which survived. No similar drop was apparent in the titer of the control. These readings were made on the fresh inactivated sera the same day they were taken. When observations on other animals failed to confirm this finding, these sera, which had been kept in the dark at ice box temperature for about a month, were reexamined, and now gave titers more nearly corresponding to those of the later experiments. The differences in titer are shown in Table I, with the record of subsequent analyses in which sera were studied while fresh, and again after standing several days. In a number of instances it will be seen that they agglutinated the Sen strain in higher dilutions on reexamination. Since this observation was made on normal as well as adrenalectomized guinea pigs the difference must be sought in some other factor, which would account for the apparent inhibition in fresh sera. Moreover, this initial inhibition does not appear with regularity. For uniformity it is necessary to use the agglutination titers from the fresh sera in drawing conclusions from the experiments, but it is apparent that disturbing factors are latent in the results.

- With the exceptions noted above, these experiments indicate that adrenalectomy subsequent to immunization has no significant effect upon the curve of typhoid agglutinin formation in guinea pigs. Similar results were found in the experiments charted in Text-fig. 2, in which, against the normal background are seen the agglutination curves of seven guinea pigs which were immunized subsequent to adrenalectomy. Their reactions to immunization fall substantially within normal limits.



..... Period before operation.
 --- One adrenal removed.
 — One, and most of the other adrenal removed.
 The shaded area covers the variations in titers of the controls.

TEXT-FIG. 1. Typhoid agglutinin titers of animals immunized before adrenalectomy.

Partial adrenalectomy, with removal of a single gland, or of one gland and as much of the other as can be taken with impunity, appears to have no influence upon the formation of typhoid agglutinins in guinea pigs.

Series II.

Cole⁷ has described a late effect of immunization which persists after demonstrable immune bodies have disappeared from the blood.

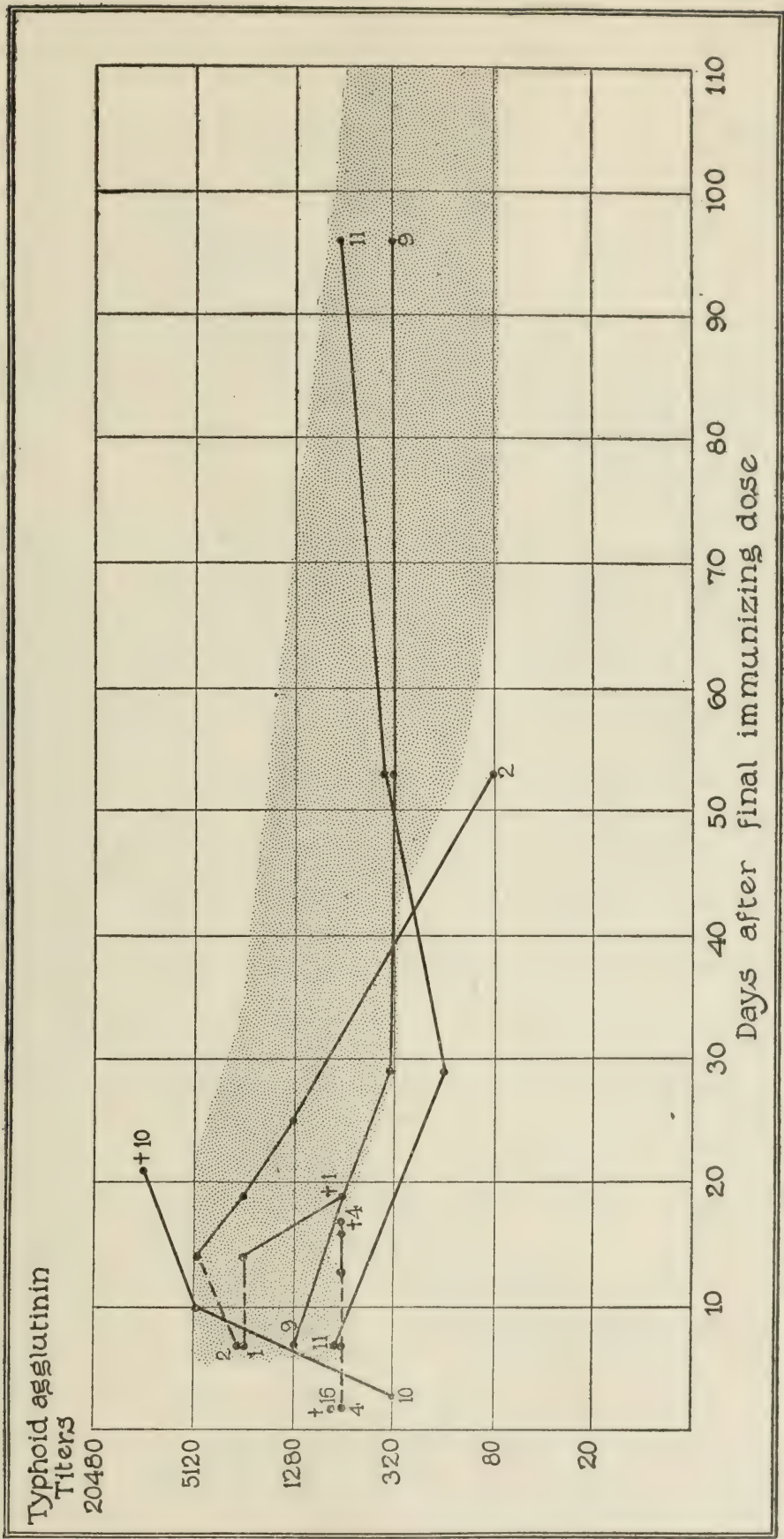
TABLE II.

Rise in Agglutinin Titers of Adrenalectomized and Control Guinea Pigs on Reinjection of Typhoid Vaccine.

Animal No.	Previous history.	Serum agglutinin titer on Mar. 5, 1917.		Serum agglutinin titer on Mar. 12, 1917.	Serum agglutinin titer on Apr. 24, 1917.
23	Adrenalectomized; immunized 84 days before.	1:160	Mar. 5, 1917. Injected intraperitoneally 0.05 mg. of typhoid vaccine.	1:320	1:80
24	Operated control; immunized 84 days before.	1:160		1:640	1:160
2	Immunized 53 days before; then adrenalectomized.	1:80		1:640	1:320
39	Normal; immunized 53 days before.	1:160		1:320	1:160
60	Normal.	0		1:20	1:20
61	"	0		0	1:10

Reinjection of previously immunized animals with a minute dose of the original antigen, an amount which has no effect upon normal animals, causes a sharp rise in the antibody curve. This effect is interpreted as due to a latent tissue sensitization. To test the presence of this phenomenon in adrenalectomized animals, two of the survivors from the experiments already described, with their controls, and with two normal guinea pigs, were injected intraperitoneally with 0.05 mg. of the typhoid vaccine used for the original immunization. At this time the animals from the earlier experiments

⁷ Cole, R. I., Experimenteller Beitrag zur Typhusimmunität, *Z. Hyg. u. Infektionskrankh.*, 1904, xlv, 371.

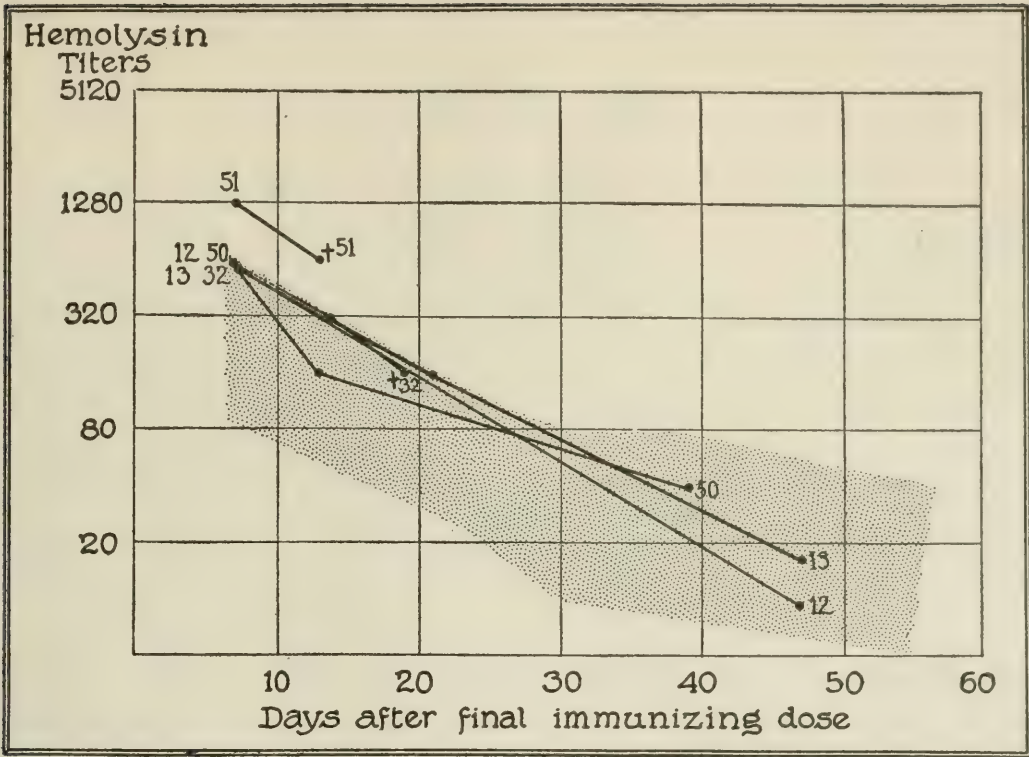


TEXT-FIG. 2. Typhoid agglutinin titers of animals immunized after adrenalectomy.

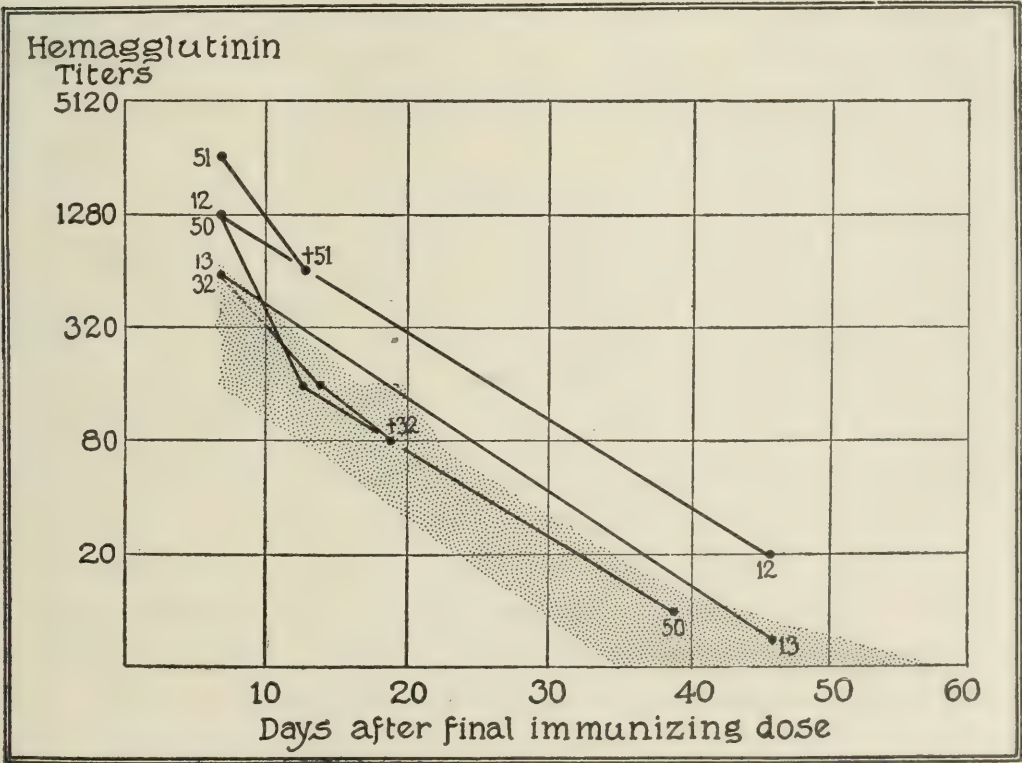
showed typhoid agglutinins in dilutions of 1:80 to 1:160 of their sera. The normal controls showed no antibodies. 7 days later the titers of the previously immunized guinea pigs had risen to 1:320 to 1:640, while the single dose of 0.05 mg. of vaccine had resulted in agglutinin formation to the extent of 1:20 in one control. These findings, and the results of a subsequent titration are found in Table II. The tissue sensitization occurred normally in the partially adrenalectomized guinea pigs and their previously immunized controls.

Series III.

With the technique already described a smaller number of experiments was made on guinea pigs immunized against hen corpuscles. Hemagglutinins and hemolysins were studied in animals adrenalectomized before or after immunization in experiments similar to those in Series I. Although the initial antibody titers corresponded roughly to those obtained with typhoid vaccine, the hemagglutinins and hemolysins disappeared from the circulating blood much more rapidly, so that the antibody content was reduced almost to nothing in the course of 2 months. The results of titrations at intervals are shown in Text-figs. 3 and 4, in which, against a background of normal variations are plotted the curves of the adrenalectomized animals. Two of these, namely Guinea Pigs 12 and 13, were adrenalectomized before immunization, losing one-half the right gland 43 days, and the left gland 7 days before the first injection of corpuscles. The other adrenalectomized animals, namely Guinea Pigs 32, 50, and 51, were first immunized and then operated upon 7 to 10 days after the final injection. The high initial agglutinin titers of Guinea Pigs 50 and 51 are due to four, instead of the usual three injections. Of the three animals in this experiment, those with the highest titers were chosen for operation. The control animal, Guinea Pig 52, had an initial hemagglutinin titer of 1:640. The slopes of the curves for Guinea Pigs 50 and 51 are seen to parallel the normal curve, although the titers for Guinea Pigs 50 and 51 do not fall within the normal limits. The charted results show that adrenalectomy has no essential influence upon the production or gradual diminution of hemolysins and hemagglutinins in guinea pigs.



TEXT-FIG. 3. Hemolysin titers of adrenalectomized animals. Guinea Pigs 12 and 13 were adrenalectomized before immunization and Guinea Pigs 32, 50, and 51 after immunization.



TEXT-FIG. 4. Hemagglutinin titers of adrenalectomized animals. Guinea Pigs 12 and 13 were adrenalectomized before immunization and Guinea Pigs 32, 50, and 51 after immunization.

Series IV.

As with the survivors from the experiments with typhoid agglutinins, the guinea pigs immunized against hen corpuscles were given a second small injection of the foreign blood cells after antibodies had almost disappeared from their sera. With two normal controls, and

TABLE III.

Rise in Hemolysin and Hemagglutinin Titers of Previously Immunized Guinea Pigs on Reinjection with Hen Corpuscles.

Animal No.	Previous operative history.	Previous immunization.	Hemolysins.			Hemagglutinins.		
			Time before injection.	Time after injection.		Time before injection.	Time after injection.	
			5 days.	7 days.	45 days.	5 days.	7 days.	45 days.
13	Double adrenalectomy 68 days before.	52 days before.	1:10	1:160	1:40	0	1:20	1:20
31	Double adrenalectomy 4 days before.	61 " "	0	1:320	1:20	0	1:40	1:10
43	Double adrenalectomy 4 days before.	52 " "	1:10	1:320	1:20	0	1:80	1:20
50	Double adrenalectomy 37 days before.	44 " "	1:40	1:320	1:40	1:10	1:40	1:40
55	Double adrenalectomy 3 days before.	40 " "	1:40	1:160	1:40	0	1:40	1:20
54	Right adrenal removed 3 days before.	40 " "	1:10	1:320		0	1:40	
33	Control operation 51 days before.	61 " "	1:40	1:160	1:40	0	1:40	1:20
44	None.	52 " "	1:10	1:160	1:40	0	1:40	0
52	"	44 " "	1:40	1:320	1:80	1:10	1:40	1:10
53	"	40 " "	1:20	1:20	1:10	0	1:20	1:10
62	None; normal.	None.	0	0	0	1:80	1:40	1:10
63	" "	"	0	0	0	0	0	0

four control guinea pigs previously immunized, six partially adrenalectomized animals were injected intraperitoneally with 0.05 cc. of washed hen corpuscles. One of the adrenalectomized guinea pigs had been operated upon before the previous immunization. The other four had lost adrenal tissue at varying intervals after immunization. Although the minute dose of antigen given did not stimu-

late antibody production to the original level, the adrenalectomized and all but one of the control animals which had been previously immunized responded with hemagglutinin titers of 1:20 to 1:80, and hemolysin titers of 1:160 to 1:320 on the 7th day, whereas the normal control animals, for which this was the first injection, failed to show demonstrable increase of antibodies. Control Guinea Pig 62 with an initial hemagglutinin titer of 1:80 before injection gave subsequent titers of 1:40 and 1:10, showing no effect from the injection. 5 weeks later the hemolysins of most of the animals had fallen below 1:80, and the hemagglutinins below 1:40. These results are given in Table III.

In addition to the experiments described above on guinea pigs, three rabbits were partially adrenalectomized and tested for *in vivo* agglutinins, as described by Bull.⁸ Typhoid bacilli injected on the 4th or the 24th day after adrenalectomy were clumped and removed from the blood stream with the same rapidity and completeness as in normal animals. The blood of these rabbits was then tested for natural typhoid agglutinins by the usual method *in vitro*. The titers ranged from 1:16 to 1:128, showing that neither adrenalectomy nor the *in vivo* agglutination had removed these antibodies from the blood.

SUMMARY.

By careful aseptic operation it was found possible to remove approximately three-quarters to seven-eighths of the adrenal tissue of guinea pigs without causing symptoms of adrenal insufficiency. Guinea pigs were immunized to *Bacillus typhosus* or to hen corpuscles at varying intervals before or after the operation, and the curves of antibody formation traced for 2 to 3 months after immunization. Comparisons with the antibody curves of control animals similarly immunized fail to show that the adrenalectomy had any influence upon the rise or persistence of antibodies in the blood.

For the purposes of the study it was not deemed necessary to produce an acute adrenal insufficiency. If the adrenal glands were the site of antibody formation or played an essential part in immunity processes, it does not seem probable that the small remainder of

⁸ Bull, C. G., The agglutination of bacteria *in vivo*, *J. Exp. Med.*, 1915, xxii, 484.

adrenal tissue left *in situ* to sustain life would affect quantitatively the antibody response to a given antigen injection as do the entire normal glands. We therefore interpret the experiments to indicate that not only are the adrenal glands not one of the important sources of typhoid agglutinins, or of hemagglutinins or hemolysins, but they play no essential part in the mechanism by which these antibodies are produced and maintained in the body.

ÆSTIVO-AUTUMNAL MALARIA. THE EXTRACELLULAR RELATION OF THE CRESCENTIC BODIES TO THE RED CORPUSCLE AND THEIR METHOD OF SECURING ATTACHMENT.*

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PLATES 33 TO 35.

(Received for publication, December 31, 1917.)

Æstivo-Autumnal Parasite.

Æstivo-autumnal parasites are extracellular throughout their life cycle; they migrate from one red corpuscle to another, destroying several in the course of their development. They pass through a sexual cycle in the human host,¹ with the formation of flagella by the microgametocyte, fertilization of the macrogamete, and its subsequent segmentation. I have seen these phases many times. The great difficulty in working out the phases in the æstivo-autumnal infections is that since the infection is usually so serious, one hardly feels justified in withholding treatment of the infected individual for the period of time necessary for the parasites to become very numerous, or, if already numerous, for the time required for a protracted study of them during their developmental phases. (The developmental and sexual phases rarely appear in the peripheral blood unless the patient has a very heavy infection.) It requires many hours for the complete examination of even one film of the patient's blood.

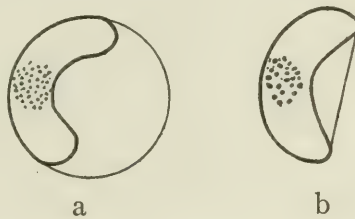
Crescentic Bodies.

The life phase of the æstivo-autumnal parasite is represented by the characteristic crescentic bodies. They develop in gradual stages

* Aided by a grant from The Rockefeller Institute for Medical Research.

¹ Rowley-Lawson, M., The æstivo-autumnal parasite: its sexual cycle in the circulating blood of man, with a description of the morphological and biological characteristics of the parasite, *J. Exp. Med.*, 1911, xiii, 263.

from the small ring-form parasites as round bodies, finally opening out into the crescent form. Several days are required for their development, which usually takes place in the internal organs of the host. The crescent may assume other forms, such as fusiform, ovoid, and round. Many of the so called round and ovoid bodies are (a) crescents viewed from their convex side; (b) crescents bent on themselves; (c) adult crescents contracted into round bodies and ovoids. The contracted round bodies and ovoids are usually seen at the edges of films made on slides and cover-slips. By the examination of many specimens one soon learns to distinguish between the developing (round body) crescent and the round form assumed by the adult crescent.

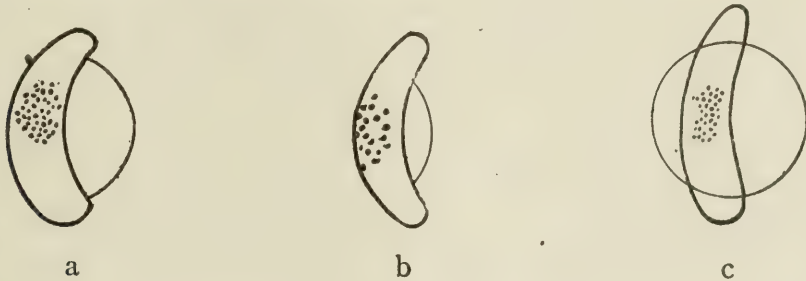


TEXT-FIG. 1, *a* and *b*. The appearances one would get if the parasite were within the corpuscle; that is, the outline of the decolorized corpuscle would correspond to the convexity of the crescentic body. One would not expect it to embrace the ends of the crescent, and then bulge out from the concavity of the crescent in the curved line, as shown in Text-fig. 2, *a*, *b*, and *c*. It would be more likely to present an appearance such as is shown in Text-fig. 1, *b*.

Extracellular Relation of the Crescent to the Red Corpuscle.—The crescentic bodies are generally believed to be within the substance of the red corpuscle in spite of the many evidences, easily demonstrated, that they are attached to its external surface. Even if one could not understand the method by which this attachment was secured, the relation of the “bib” to the parasite should enable one to see that they are not within the corpuscle. For instance, if they were within the corpuscle, one would expect to get such appearances as are shown in Text-fig. 1. As a matter of fact, the appearances most commonly met with and illustrated are those given in Text-fig. 2.

Attachment of the Crescent to the Red Corpuscle.—The crescent follows in general the same method of attachment to the corpuscle as

do the parasites of the other malarial infections.² They encircle with their cytoplasm mounds of hemoglobin substance, which assists them to maintain their rather precarious position on the surface of the corpuscle while they dissolve and digest the hemoglobin. The mounds of hemoglobin to which the corpuscles are attached may be seen protruding through the cytoplasm of the body proper of the crescent as well as at the periphery (Figs. 1 to 105). One might say that the parasite attaches itself to the corpuscle in two ways: (a) encircling with its body protoplasm surface mounds of hemoglobin; (b) encircling with pseudopodia arising from the cytoplasm peripheral mounds of hemoglobin.



TEXT-FIG. 2, *a*, *b*, and *c*. The crescent wraps itself around the red corpuscle and proceeds to decolorize it. The so called "bib" is the decolorized corpuscle. When the bib is present it practically always comes from the concavity of the crescentic body as shown in *a*, *b*, and *c*. Ordinarily the bib appears only on one side of the crescent, but occasionally it may be seen on both sides as is schematized in *c*. In these instances the outline of the decolorized corpuscle can usually be traced through the substance of the crescent.

Surface Mounds.—The mounds of hemoglobin substance protruding through the protoplasm of the parasite do not seem to alter the general outline of the crescentic body. In many instances where the apex of the surface mound extends beyond the periphery of the crescentic body, the outline of the crescent may be traced beneath the transparent mound. The mounds protrude through various parts of the body of the crescent, some of them protruding where the nucleoplasm is supposed to be; in fact, in rare cases the chro-

² In previous publications I have explained and illustrated the method by which the young parasites of æstivo-autumnal infections (Figs. 1 to 7), as well as the parasites of tertian infections (Figs. 8 to 10), secure their attachment to the external surface of red corpuscles.

matin granules may be seen outlining the hemoglobin mound at its base (Figs. 21 and 79 at oo). Frequently the pigment granules may be seen outlining the base of a hemoglobin mound (Figs. 11, 17, and 44 at x).

Peripheral Mounds.—These are seen along the edges of the crescentic body, and do not appear to have protruded through the body proper of the parasite. I believe that these peripheral mounds are encircled by pseudopodia.

Attaching Pseudopodia.—I do not know whether these pseudopodia are used for the purpose of capturing their prey as well as for the purpose of securing it after it has been captured. They arise from the cytoplasm of the parasite, staining similarly, and may show definitely, especially in specimens where the hemoglobin mounds are seen. As one would expect, considering their purpose, they may be seen either in the form of loops (Figs. 43, 58, 60, 70, 98, and 105 at o), or as strings of cytoplasm (Figs. 21, 34, 56, 57, 59, 79, 97, and 98 at o). The large amount of cytoplasm which may enter into the formation of these pseudopodia is surprising (Figs. 59, 60, 70, 98, 99, and 105).

I have observed these mounds, especially the peripheral mounds, in fresh preparations. This has led me to believe that perhaps some of the small round bodies seen by observers about the periphery of the crescent might be hemoglobin mounds.

Celli and Guarnieri³ note that "these crescent and ovoid forms may show small round bodies—buds, as it were—about the periphery, one or more in number." Thayer⁴ states: "We may observe in certain instances the protrusion of small delicate, bud-like bodies which are cut off from the cell." Celli and Guarnieri suggest that these bodies may represent a method of reproduction, while Thayer states that it is probably a degenerative process. Sforza,⁵ judging from certain observations on the staining reactions of crescents, concludes that "the greater part of the crescentic body is nothing more or less than the degenerate red corpuscle." To reach this conclusion, could he have seen the hemoglobin mounds protruding through the crescentic body?

³ Celli and Guarnieri, quoted from Thayer, W. S., and Hewetson, J., *The malarial fevers of Baltimore, Johns Hopkins Hosp. Rep.*, 1895, v, 162.

⁴ Thayer, W. S., *Lectures on the malarial fevers*, New York, 1897, 73.

⁵ Sforza, quoted from Thayer and Hewetson, *The malarial fevers of Baltimore, Johns Hopkins Hosp. Rep.*, 1895, v, 169.

*Decolorization of Corpuscular Mounds by Parasitic Action.
Vacuolization.*

As a rule, mounds of hemoglobin substance are rather rapidly decolorized (dissolved and assimilated) by the parasite. This may be demonstrated by the examination of a large series of films taken in immediate succession. After decolorization of the surface mounds has taken place, the appearance of the parasite corresponds to what has often been described and pictured as "vacuolization." Mounds in the process of decolorization by the action of the parasite are frequently seen (Figs. 62 to 69). I have observed vacuoles in the living parasite many times and have never considered the process a degenerative one. The vacuoles vary in size and shape and two or more may run together to make one.

The idea that vacuolization is synonymous with degeneration seems to be well fixed in the minds of many observers. Canalis⁶ pictures a crescent with vacuoles, describing it as a crescent showing degeneration. Antolisei and Angelini⁷ describe "degenerate vacuolating forms which represent the death of the parasite." Mannaberg⁸ writes: "In fresh preparations appearances are sometimes seen in the crescents which must be considered to be processes of degeneration. They consist of the appearance of clear circles and spots, which alter their shape under the observer's eye." Marchiafava and Bignami⁹ write: "We may also see the process of vacuolization of the crescent bodies as well as of the ovoid and round ones," describing it as a degenerative alteration. Celli and Guarnieri³ note "vacuolic degeneration of the crescentic forms." Manson¹⁰ writes that the protoplasm of the crescent "shows vacuolation and other signs of degeneration."

⁶ Canalis, P., Studi sulla Infexione malarica Sulla varietà parassitaria delle forme semilunari di Laveran e sulle febbri malariche che da esse dipendono, *Arch. sc. med.*, 1890, xiv, 75, and Plate C, Fig. 11.

⁷ Antolisei and Angelini, quoted from Thayer and Hewetson, The malarial fevers of Baltimore, *Johns Hopkins Hosp. Rep.*, 1895, v, 164.

⁸ Mannaberg, J., The malarial parasites. A description based upon observations made by the author and other observers; Translation by Felkin, R. W., London, 1894, 287.

⁹ Marchiafava, E., and Bignami, A., Malaria, in Stedman, T. L., Twentieth century practice, New York, 1900, xix, 47.

¹⁰ Manson, P., Tropical diseases: a manual of the diseases of warm climates, London, Paris, New York, and Melbourne, 2nd edition, 1900, 14.

Thayer¹¹ states: "Vacuolization of the crescentic, ovoid, and round bodies is not very uncommon. This is usually associated with a diminution of the refractiveness of the parasite and often with a loss of regular outline. The vacuoles are small, but may vary considerably in size, sometimes becoming confluent and larger. The process is evidently degenerative." Thayer and Hewetson¹² state that the "vacuolic degeneration" of crescentic forms is a process which has previously been described by Laveran.

Vacuoles.

Contractile or Pulsatile Vacuoles.—In protozoa the vacuole is not a vacant space. In the fresh water amebæ, in addition to food vacuoles, one may see contractile or pulsatile vacuoles. These vacuoles are exceptional in size and constancy of position. They are usually excretory organs, containing a combination of fluid and gas. At fairly regular intervals they may be seen to contract until they disappear, reforming slowly. But it is not the contractile or pulsatile vacuoles with which we have to do in connection with the malarial parasite, but with the food vacuoles.

Food or Nutritive Vacuoles.—These are usually regarded as of temporary character. They contain liquid, not gas, probably a chemical ferment which dissolves the hemoglobin and makes it available for utilization by the parasite. The parasite assimilates what it needs for nutrition, the waste products being converted into pigment, which is excreted when the parasite segments.

The fresh water ameba resembles the malarial ameba in some respects. It secures its prey by means of pseudopodia which surround the prey, the pseudopodium of the ameba uniting to enclose it within the boundary of its protoplasm, in this way forming a so called nutritive vacuole with the prey as the inclusion. The ameba then proceeds to dissolve the inclusion, absorbing the dissolved material into its substance, storing the reserves, and throwing off the waste products.

I have seen much the same process occur with the mononuclear leukocyte of human blood.¹³ The leukocyte put out pseudopodia from

¹¹ Thayer, Lectures on the malarial fevers, New York, 1897, 72.

¹² Thayer and Hewetson, The malarial fevers of Baltimore, *Johns Hopkins Hosp. Rep.*, 1895, v, 162.

¹³ Rowley, M. W., A fatal anæmia with enormous numbers of circulating phagocytes, *J. Exp. Med.*, 1908, x, 78.

its cytoplasm, which captured and included a polynuclear leukocyte, the pseudopodia uniting to form the wall of the nutritive vacuole with the polynuclear leukocyte within it. The mononuclear cell then proceeded to dissolve the inclusion and the gradual disappearance of the structure of the polynuclear leukocyte could be watched.

The vacuoles seen in connection with the malarial parasite are what one would expect to find and are not an indication of degeneration. Grassi¹⁴ shows them in crescentic bodies, Schaudinn¹⁵ pictures vacuoles in the "ookinete," and Ruge¹⁶ illustrates them in a proteosoma. Even the decolorized mound ("achromatic area") seen in connection with the young parasite must be a converted nutritive vacuole since the parasite has dissolved and assimilated the hemoglobin which was enclosed within its pseudopodium.

Is it possible that the malarial parasite, like the fresh water ameba, may secrete reserves which would enable it to withstand lack of food for a short time? If this were so, it would suggest what we already know to be necessary—vigorous and long continued treatment.

SUMMARY.

Æstivo-autumnal parasites, including the crescentic bodies, are always extracellular; that is, they are attached to the external surface of the red corpuscles.

Crescentic bodies attach themselves to the red corpuscles just as the younger parasites do, by encircling, with their cytoplasm, mounds of hemoglobin substance. These hemoglobin mounds may be seen protruding through various portions of the crescentic bodies, as well as at the periphery of the parasites. The base of the mounds is occasionally outlined by the chromatin or pigment granules.

The hemoglobin mounds protruding through the body proper of

¹⁴ Grassi, B., *Die Malaria*, Studien eines Zoologen, Jena, 2nd edition, 1901, Figs. 11, 12, 14 to 17, 20, 21, 24, 25, and 28.

¹⁵ Schaudinn, F., *Studien über krankheitserregende Protozoen. II. Plasmodium vivax* (Grassi & Feletti), der Erreger des Tertianfiebers beim Menschen, *Arch. f. Protistenkunde*, 1903, xix, 169, and Plate 4, Figs. 37, 38, 39, and 40.

¹⁶ Ruge, R., *Einführung in das Studium der Malaria-krankheiten mit besonderer Berücksichtigung der Technik. Ein Leitfaden für Schiffs- und Colonialärzte*, Jena, 1901, Plate 1, Fig. 41.

the crescentic bodies do not seem to alter the general outline of the parasites. The outline of the parasites may be traced through the transparent mounds.

Whenever attaching pseudopodia are observed they are seen to arise from the cytoplasm of the parasites and may be in the form of loops or strings.

When the crescents are attached they proceed to dissolve the hemoglobin to make it available for utilization, assimilating what is required for nutrition, the waste product being in the form of pigment granules.

After the hemoglobin mounds, to which the crescents are attached, have been decolorized by parasitic action, an appearance is obtained which has been described by most observers as vacuolization of the crescentic body. These observers believe the picture to be one of degeneration.

The decolorized mounds or vacuoles ("achromatic areas") seen in connection with malarial parasites correspond to the nutrition vacuoles of the common amebæ, and possibly the malarial parasite may, like these amebæ, secrete reserve food.

EXPLANATION OF PLATES.

PLATE 33.

Magnification, $\times 1,690$.

FIGS. 1 to 7. Young æstivo-autumnal parasites attached to peripheral mounds of hemoglobin substance. The pseudopodia of the parasites have encircled the mounds at their base.

FIGS. 8 to 10. Adult tertian parasites attached to peripheral corpuscular mounds.

FIGS. 11 to 35. Crescentic bodies of æstivo-autumnal infections attached to peripheral and surface mounds of hemoglobin substance. In Figs. 11, 17, 26, and 28 the pigment granules are seen outlining the hemoglobin mounds at their base, at x. In Fig. 21 the chromatin granules may be seen outlining the base of the mound at oo. In Figs. 21 and 34 the pseudopodium may be seen at o.

Figs. 12, 13, 14, 15, 16, 18, 19, 20, 21, 22, 24, 25, 26, 27, 28, 29, 30, 31, 33, and 35 correspond to Figs. 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, and 90.

Examination of these pictures with a magnifying glass will show definitely the mounds of hemoglobin substance protruding through the bodies of the crescents.

PLATE 34.

Magnification, $\times 1,690$.

FIGS. 36 to 55. Crescentic bodies attached to peripheral and surface mounds of hemoglobin substance. Fig. 43 shows at o the pseudopodium in the form of a loop. Fig. 44 shows at x pigment granules surrounding the base of the hemoglobin mound. Fig. 45 shows at xx mounds of hemoglobin substance, which was stained a deep pink, reproducing black in the photograph.

Figs. 37, 41, 44, 46, 49, and 55 correspond to Figs. 91, 92, 93, 94, 95, and 96.

FIG. 56. A crescent body attaching to a red corpuscle by means of its pseudopodia, seen at o. This figure corresponds to Fig. 97.

FIG. 57. A crescent attached to a partially decolorized corpuscle. The mounds of hemoglobin substance may be seen and a pseudopodium is seen at o.

FIG. 58. A crescent with an attaching loop of cytoplasm showing at o.

FIG. 59. A crescentic body showing corpuscular mounds and an attaching pseudopodium at o. This figure corresponds to Fig. 98.

FIG. 60. The attaching pseudopodia may be seen at o in the form of loops. This figure shows a large amount of cytoplasm entering into the pseudopodia. This figure corresponds to Fig. 99.

FIG. 61. The corpuscle to which the parasite is attached shows well here, and a careful examination of the parasite will show the surface mounds. This figure corresponds to Fig. 100.

FIG. 62. Here the hemoglobin mounds are showing decolorization. The entire upper end of the parasite is occupied by a partially decolorized mound of hemoglobin protruding through the cytoplasm of the parasite.

FIG. 63. Here one sees the beginning of what is popularly termed vacuolization. The parasite is dissolving and digesting the hemoglobin and the digestive vacuoles can be seen more clearly than when they are filled with the well stained hemoglobin substance. This figure corresponds to Fig. 101.

FIGS. 64 and 65. The dissolving of the hemoglobin in the vacuole, the operation being a little more advanced in Fig. 65. Fig. 64 corresponds to Fig. 102.

FIG. 66. The strands of cytoplasm of the parasite may easily be seen between the vacuoles. The vacuoles are oval in shape and the included hemoglobin almost decolorized. This figure corresponds to Fig. 103.

FIGS. 67 and 68. Definite vacuoles in the body of the parasites. Fig. 68 corresponds to Fig. 104.

FIG. 69. A large vacuole in the parasite.

FIG. 70. The crescentic body here shows that it is attached to corpuscular mounds and a loop arrangement of cytoplasm is seen extending from one end of the parasite to the other end, at o. This figure corresponds to Fig. 105.

PLATE 35.

Magnification, $\times 1,690$.

FIGS. 71 to 105. These pictures are colored photographs of certain parasites shown in the black and white reproductions. They show the parasites attached to peripheral and surface mounds of hemoglobin substance. The attaching pseudopodia and the nutritive vacuoles of the parasites can also be seen.

Fig. 71 corresponds to Fig. 12.

Fig. 72 corresponds to Fig. 13.

Fig. 73 corresponds to Fig. 14.

Fig. 74 corresponds to Fig. 15.

Fig. 75 corresponds to Fig. 16.

Fig. 76 corresponds to Fig. 18.

Fig. 77 corresponds to Fig. 19.

Fig. 78 corresponds to Fig. 20.

Fig. 79 corresponds to Fig. 21.

Fig. 80 corresponds to Fig. 22.

Fig. 81 corresponds to Fig. 24.

Fig. 82 corresponds to Fig. 25.

Fig. 83 corresponds to Fig. 26.

Fig. 84 corresponds to Fig. 27.

Fig. 85 corresponds to Fig. 28.

Fig. 88 corresponds to Fig. 29.

Fig. 87 corresponds to Fig. 30.

Fig. 86 corresponds to Fig. 31.

Fig. 89 corresponds to Fig. 33.

Fig. 90 corresponds to Fig. 35.

Fig. 91 corresponds to Fig. 37.

Fig. 92 corresponds to Fig. 41.

Fig. 93 corresponds to Fig. 44.

Fig. 94 corresponds to Fig. 46.

Fig. 95 corresponds to Fig. 49.

Fig. 96 corresponds to Fig. 55.

Fig. 97 corresponds to Fig. 56.

Fig. 98 corresponds to Fig. 59.

Fig. 99 corresponds to Fig. 60.

Fig. 100 corresponds to Fig. 61.

Fig. 101 corresponds to Fig. 63.

Fig. 102 corresponds to Fig. 64.

Fig. 103 corresponds to Fig. 66.

Fig. 104 corresponds to Fig. 68.

Fig. 105 corresponds to Fig. 70.

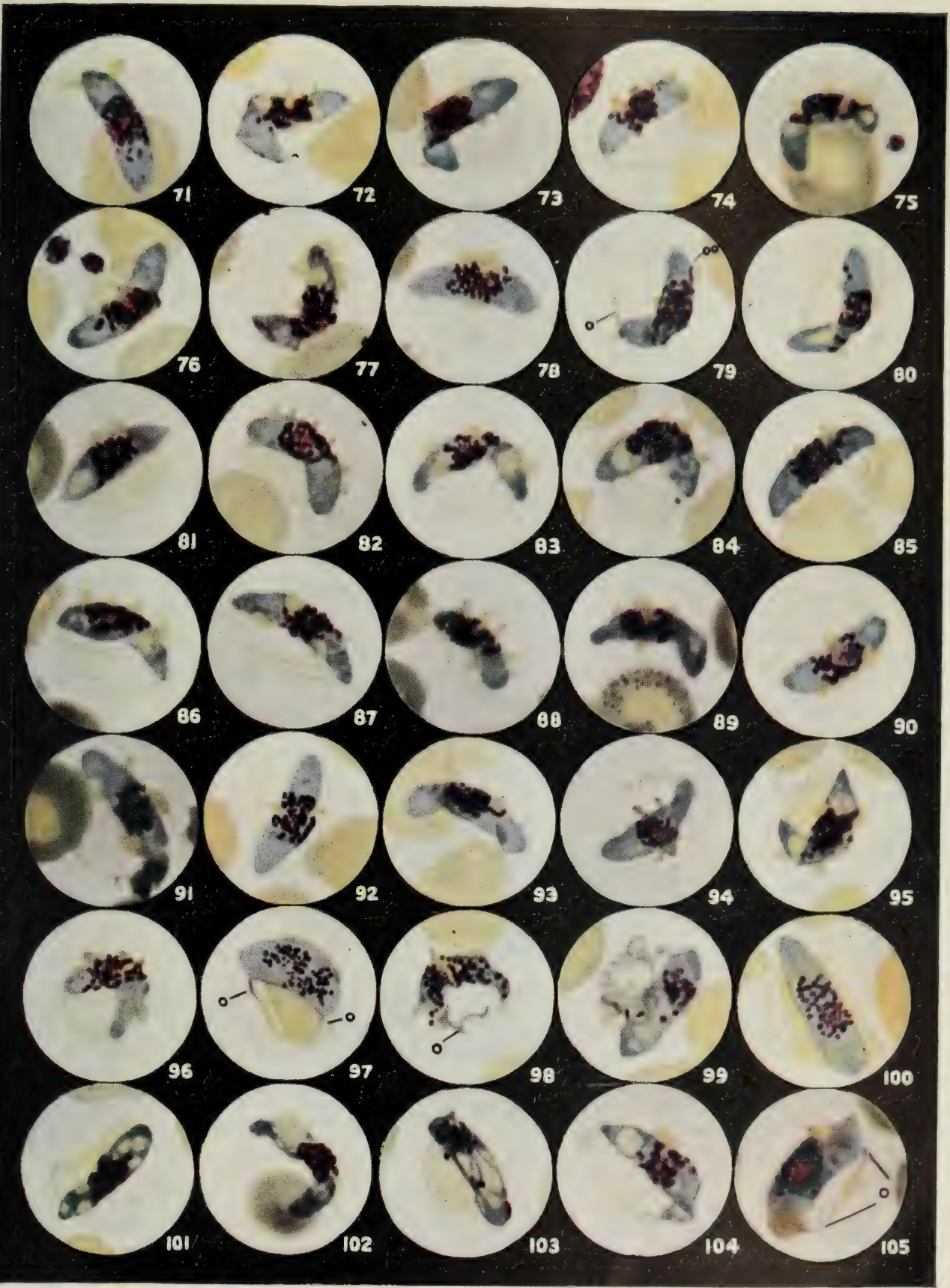


(Lawson: Crescentic bodies in astivo-autumnal malaria.)





(Lawson: Crescentic bodies in æstivo-autumnal malaria.)



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ÆSTIVO-AUTUMNAL PARASITES. MULTIPLE INFECTION OF RED CORPUSCLES AND THE VARIOUS HYPOTHESES CONCERNING IT.*

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PLATES 36 TO 39.

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Malarial Parasites.

There are three species of malarial parasites, each with its special morphological and biological characteristics. They are: (1) tertian parasites, (2) quartan parasites, and (3) æstivo-autumnal parasites. It is with the last variety that this paper deals.

Æstivo-Autumnal Parasite.—The young forms of these parasites are very much like those of the tertian and quartan infections, except that, as a rule, the parasites of the æstivo-autumnal infections are relatively smaller than those of the tertian and quartan infections. They are more delicate in appearance, have a more clean-cut outline and a smaller chromatin mass. One should learn to distinguish these morphological differences, as the young parasite may be the only form present at the time of the examination of the blood. The æstivo-autumnal parasites produce the gravest manifestations of the malarial infections so that an early diagnosis may be of the greatest importance.

Multiple Infection of Red Corpuscles with Young Parasites.

Multiple infection of red corpuscles with young parasites is seen in all malarial infections and it is not a rare occurrence in the æstivo-autumnal infections. In films from the circulating blood I have seen from two to seven young parasites on a corpuscle (Figs. 1 to 75).

* Aided by a grant from The Rockefeller Institute for Medical Research.

The occurrence is always accidental and has no significance other than that, if the instances are numerous, it usually means a heavy infection. As a rule, the number of parasites attached to individual corpuscles increases in direct proportion to the severity of the infection; therefore one would not expect to find three, four, or five parasites on a corpuscle in a film where the parasites present were few in number. This fact in itself should suggest that instances of multiple infection have no significance; but various theories have been formulated to explain certain examples.

Theories as to Multiple Infection.—The position of the attached parasites in relation to each other has, apparently, suggested certain theories, such as conjugation ("syngamy," "cytogamy"). The fusion of two cells, cytoplasm to cytoplasm, and chromatin to chromatin, to form a new individual, is a process which has been described frequently in connection with certain protozoa.

Mannaberg¹ was one of the first observers to formulate a theory as to the conjugation of the young amebæ of æstivo-autumnal infections. Two or more parasites were seen attached to adjacent hemoglobin mounds. They were attached so closely together that a portion of the cytoplasm of one parasite was overlying a portion of the cytoplasm of the adjacent parasite. This appearance (Figs. 11 to 30 and 119 to 125) was interpreted by Mannaberg as conjugation. He believed that a fusion of the cytoplasm had taken place, resulting in transitional forms in the formation of the crescents. It would seem that it did not make any difference whether two, three, or four of these young parasites had united to form a crescent, for he states: "I have observed two, or more rarely three, of these parasites may lie closely adhering to one another," and he speaks of "these conglomerate parasites, consisting of two to four specimens." Wright² states: "My observations appear to support those of Mannaberg in regard to the genesis of the crescent—the syzygium—from a corpuscle doubly infected by parasites," and "In the stained specimens the syzygies appear to be in the act of conjugation."

As a matter of fact these parasites never unite. The same appearance may be seen in tertian infections.

¹ Mannaberg, J., *The malarial parasites. A description based upon observations made by the author and other observers; Translation by Felkin, R. W., London, 1894, 289.*

² Wright, H., *The malarial fevers of British Malaya, Studies from Institute for Medical Research, Federated Malay States, Singapore, 1901, i, 4.*

Multiple Infection of Corpuscular Mounds.

I have seen from two to five young parasites attached to one corpuscular mound (Figs. 1 to 9, 19, 20, 24, 25, 27, 29 to 35, and 37 to 70). Multiple infections of mounds have no significance. The heavier the infection, the more frequently it is seen. The parasites attached to one mound may be in similar or in varying stages of development. The cytoplasm of each parasite is in contact with the hemoglobin mound, and each parasite has a share in its destruction.

Occasionally one finds two parasites attached to one mound so as to give the appearance of a developing crescent (Figs. 31 to 35, 126, and 127), and the same appearance may be seen in tertian infections (Fig. 35). In comparing these instances of æstivo-autumnal and tertian infections of mounds, note the relatively larger size of the chromatin masses of the tertian parasites.

Many theories have been formulated as to the significance of the appearance where two or more parasites encircled one mound of hemoglobin substance, especially by observers who believed the parasites to be within the substance of the corpuscle.

The youngest form of the æstivo-autumnal parasite to attach itself to the red corpuscle may require its entire cytoplasm to encircle a hemoglobin mound. When one of these parasites is so attached, it appears as a ring-form of a delicate, thread-like structure, more or less uniform in size throughout its circumference. If two or more of these tiny parasites encircle the one hemoglobin mound, the cytoplasm of one parasite superimposed over the cytoplasm of the other parasite or parasites, and the chromatin masses separated or lying close together, as accident may direct, the appearance of a single ring with more than one mass of chromatin is obtained (Figs. 1 to 4 and 6).

Marchoux³ suggested that these forms result from conjugation, and Ewing⁴ states that while such an explanation appears reasonable, it is without proof, and the more probable explanation is the incomplete fusion of the chromatin in the rosette.

³ Marchoux, E., *Le paludisme au Sénégal*, *Ann. Inst. Pasteur*, 1897, xi, 647.

⁴ Ewing, J., *Malarial parasitology*, *J. Exp. Med.*, 1900-01, v, 482.

As the æstivo-autumnal parasite increases in size, especially if it has developed a thickening of one segment, giving what has been called the "signet-ring" appearance, it is easier to recognize the individual parasites when two or more encircle one mound (Figs. 31 to 34, 36 to 70, 106 to 112, 114 to 118, and 126 to 129).

Craig⁵ but follows in the footsteps of the early investigators. He interprets parasites attached to adjoining mounds and parasites attached to one mound as conjugation forms. The theory of conjugation as advanced by him may be summed up in a few words. He states that it occurs "within the infected erythrocytes,"⁶ that conjugation "occurs only between two young hyaline forms of the plasmodia, indistinguishable in size and structure,"⁷ that it "is completed during the hyaline stage before the formation of pigment,"⁸ that the "process occurs in every malarial infection in which quinine has not been given early,"⁹ and that it is "the most rational explanation of latency and recurrence in malarial disease."⁹ In the same article Craig illustrates¹⁰ two young parasites side by side, a portion of the cytoplasm of one parasite overlying a portion of the cytoplasm of the other¹ parasite. In referring to them he states: "Protoplasmic union is almost complete, and the portions in apposition are beginning to be absorbed." He also pictures¹¹ two young parasites encircling one corpuscular mound, interpreting the appear

⁵ Craig, C. F., Studies in the morphology of malarial plasmodia after the administration of quinine, and in intracorpuseular conjugation, *J. Infect. Dis.*, 1910, vii, 285, 318.

⁶ Craig, Studies in the morphology of malarial plasmodia after the administration of quinine, and in intracorpuseular conjugation, *J. Infect. Dis.*, 1910, vii, 300.

⁷ Craig, Studies in the morphology of malarial plasmodia after the administration of quinine, and in intracorpuseular conjugation, *J. Infect. Dis.*, 1910, vii, 304.

⁸ Craig, Studies in the morphology of malarial plasmodia after the administration of quinine, and in intracorpuseular conjugation, *J. Infect. Dis.*, 1910, vii, 309.

⁹ Craig, Studies in the morphology of malarial plasmodia after the administration of quinine, and in intracorpuseular conjugation, *J. Infect. Dis.*, 1910, vii, 301.

¹⁰ Craig, Studies in the morphology of malarial plasmodia after the administration of quinine, and in intracorpuseular conjugation, *J. Infect. Dis.*, 1910, vii, Fig. 5.

¹¹ Craig, Studies in the morphology of malarial plasmodia after the administration of quinine, and in intracorpuseular conjugation, *J. Infect. Dis.*, 1910, vii, Fig. 7.

ance as "a conjugation form in which the two chromatin masses are distinct and the portions of protoplasm in apposition have become absorbed, resulting in the formation of a large ring-like body with two chromatin masses."¹² In his diagrammatic figures Craig advances the stages in his conjugation theory by bringing the chromatin masses closer and closer together until they are side by side. He finally pictures a large ring-form parasite with one mass of chromatin, which he assumes to be "a form resulting after conjugation is completed"¹³

Premature Division of the Chromatin.

In the adult parasite nuclear division seems to go on more rapidly than cytoplasmic, and it may be completed before cell division takes place. But premature division of the chromatin never takes place in the young parasite.

Certain observers have described what they believed to be a precocious division of the chromatin. Ziemann¹⁴ was at first uncertain whether the appearance was due to two fused parasites or to a precocious division of one nucleus, but finally accepted the latter hypothesis, describing "the separation of one, or rarely two, accessory granules from the original mass in cells infected by single parasites. Sometimes the accessory granule was much smaller than, sometimes nearly as large as, the main granule." And Ewing¹⁵ goes on to say: "All of these appearances I have seen in single parasites, less often in single members of conjugating pairs, and I agree with Ziemann as to their significance." Emin¹⁶ gives examples of two and three parasites encircling one corpuscular mound, and interprets the condition as that of precocious division of the chromatin.

Variation in the size of the chromatin masses of young parasites is frequently observed. It may be only an apparent variation in size, or a breaking up of the chromatin due to technique, or it may be a normal occurrence. The nuclei of young parasites may appear

¹² Craig, Studies in the morphology of malarial plasmodia after the administration of quinine, and in intracorpuseular conjugation, *J. Infect. Dis.*, 1910, vii, 308.

¹³ Craig, Studies in the morphology of malarial plasmodia after the administration of quinine, and in intracorpuseular conjugation, *J. Infect. Dis.*, 1910, vii, 318.

¹⁴ Ziemann, H., quoted from Ewing, Malarial parasitology, *J. Exp. Med.*, 1900-01, v, 479.

¹⁵ Ewing, Malarial parasitology, *J. Exp. Med.*, 1900-01, v, 479.

¹⁶ Emin, A., Une variété nouvelle du parasite de Laveran, *Bull. Soc. path. exot.*, 1914, vii, 385; Figs. 3 to 5.

to be larger in certain parts of a film, where the red corpuscles are thinly spread, than they do in the thicker portions. This appearance is due to the flattening of the chromatin mass with consequent enlargement. Irregularity in the size and distribution of chromatin masses in the young parasite is frequently due to technique while spreading the film and illustrates how easily the chromatin may be subdivided. Occasionally young parasites in varying stages of development may occupy one corpuscular mound (Figs. 2, 8, 9, 49, 62, 65, 68 to 70, 112, and 114 to 116). In these instances the cytoplasm may vary in amount and the chromatin mass in size. Where several young parasites are attached to a corpuscle the same variation may be seen (Figs. 26, 73 to 75, 117, and 118). Variations in the size of young parasites are to be expected since all adult parasites do not segment at once in any malarial infection.

Multiple Infection of Red Corpuscles with Crescentic Bodies.

This occurrence (Figs. 77 to 105 and 131 to 140), like multiple infection by young parasites, is always accidental. It has no significance other than the fact that, if several instances are seen in one film, a severe infection is indicated. So far as I know, there have been no theories advanced to explain the occurrence of more than one crescent on a corpuscle. I have seen three crescents on a corpuscle (Fig. 100), but rarely. Two is the number usually seen and I believe that two on a corpuscle are considered a rare occurrence.

There is apparently very little literature referring to two crescents on a corpuscle. Marchiafava and Bignami¹⁷ write: "We have also seen two crescents within the same blood corpuscle, the curved portions being face to face." Manson¹⁸ figures two crescents attached to the one corpuscle, and states: "Very rarely twin or double crescents, that is two crescents in one corpuscle—are encountered." Cropper¹⁹ pictures two crescents on a corpuscle.

¹⁷ Marchiafava, E., and Bignami, A., *Malaria*, in Stedman, T. L., *Twentieth century practice*, New York, 1900, xix, 42.

¹⁸ Manson, P., *Tropical diseases: a manual of the diseases of warm climates*, London, Paris, New York, and Melbourne, 2nd edition, 1900, 14.

¹⁹ Cropper, J., Phenomenal abundance of parasites in a fatal case of pernicious malaria, *Lancet*, 1908, ii, 16.

In my experience, one of the rarest occurrences in instances of multiple infection of red corpuscles is the presence of a young parasite and a crescent (Figs. 76 and 130). I have seen but three examples of this.

I am convinced that malarial parasites do not conjugate.

Marchiafava and Bignami²⁰ state: "Not infrequently several young parasites are seen in the same red corpuscle; we have counted up to six or seven, and when they are very close together they may appear to be intimately adherent. . . . we cannot hold it to have been conclusively demonstrated that the young parasites collected within one red corpuscle become merged together; on the contrary, they follow their own development."

Many of the morphological and biological phases would cease to be obscure if observers realized that malarial parasites are attached to the external surface of red corpuscles.

SUMMARY.

1. Multiple infection of red corpuscles with young parasites is seen in all malarial infections, but it is found most frequently in the æstivo-autumnal infections. The occurrence is accidental and has no significance other than that if the instances are numerous it suggests a heavy infection.

2. In instances of multiple infection the young parasites may be seen to be attached: (a) each encircling its own corpuscular mound, giving the typical ring-form picture, or (b) two or more encircling one corpuscular mound, giving the appearance of a single ring with two or more masses of chromatin.

3. Certain hypotheses as to the conjugation of malarial parasites have been formulated by observers to explain various instances of multiple infection. I do not believe that conjugation ever occurs. I believe that these hypotheses resulted from observation of certain appearances presented by the attached parasites, as when they are attached so closely together that they may appear to be adherent, or when two or more are attached to one corpuscular mound, giving

²⁰ Marchiafava and Bignami, *Malaria*, in Stedman, *Twentieth century practice*, New York, 1900, xix, 46.

the appearance of a single parasite with more than one mass of chromatin.

4. Certain appearances have also been described as a precocious division of the chromatin masses of young parasites. In these instances the chromatin granules were usually described as varying in size. Such an appearance may be explained as follows: (a) two young parasites in varying stages of development may encircle one corpuscular mound, the cytoplasm of one parasite being superimposed over that of the other parasite, giving a picture of a single ring with two unequal masses of chromatin; or (b) the variation in the size and number of the chromatin masses may be the result of traumatism, as the nuclei of young parasites are rather easily broken up.

5. Multiple infection of red corpuscles with crescentic bodies is considered rather a rare occurrence. It is always accidental, and if the instances are numerous it means a severe infection.

6. When one accepts the fact that all malarial parasites are attached to the external surface of the red corpuscles, the biological and morphological characteristics of the parasites cease to be obscure.

EXPLANATION OF PLATES.

PLATE 36.

ÆSTIVO-AUTUMNAL PARASITES (TERTIAN PARASITES, FIG. 35).

Magnification, $\times 1,840$.

FIG. 1. Two very young parasites encircling one surface hemoglobin mound. The cytoplasm of one parasite superimposed over that of the other parasite gives the appearance of a single ring with two chromatin masses. One chromatin mass extends beyond the periphery of the corpuscle.

FIG. 2. Examples of two young parasites encircling one surface hemoglobin mound. The chromatin masses of these parasites vary in size.

FIG. 3. Two young parasites encircling a peripheral hemoglobin mound.

FIG. 4. Three young parasites are attached to this corpuscle; two of them encircle one surface hemoglobin mound at x. Pigment granules are seen at o.

FIG. 5. Two young parasites attached to one surface hemoglobin mound. The nuclei of these parasites are shaped to the mound.

FIG. 6. Three young parasites encircling one surface hemoglobin mound. The parasite at x appears to be more advanced in development than the other two.

FIG. 7. Three young parasites encircling one decolorized hemoglobin mound.

FIG. 8. Four young parasites encircling one surface hemoglobin mound. The chromatin masses vary in size. This figure corresponds to Fig. 112.

FIG. 9. Four young parasites encircling one surface hemoglobin mound. The chromatin masses vary in size.

FIG. 10. Five young parasites are attached to this corpuscle; four of them encircle one surface hemoglobin mound. A pigment granule may be seen at o. This figure corresponds to Fig. 113.

FIGS. 11 to 15. These parasites have developed a thickening of one segment. They encircle, in various positions, adjacent hemoglobin mounds. They are attached so closely together that a portion of the cytoplasm of one parasite is overlying a portion of the cytoplasm of the parasite attached to the adjacent mound (corresponding to Mannaberg's conjugation forms). Figs. 11, 13, and 14 show decolorized hemoglobin mounds in connection with but one of the two parasites, suggesting that one of these parasites attached itself to the red corpuscle before the other parasite did. Pigment granules may be seen at o. Fig. 12 corresponds to Fig. 120.

FIG. 16. Two young parasites encircling one peripheral hemoglobin mound. The apex of the mound is not yet decolorized.

FIGS. 17 to 19. Young parasites are shown encircling adjacent decolorized hemoglobin mounds. In Fig. 17 a pigment granule in connection with one parasite may be seen at o.

FIG. 20. At x two young parasites encircle one hemoglobin mound, and at o the two young parasites are attached to adjacent mounds, a portion of the cytoplasm of one overlying a portion of the cytoplasm of the other parasite.

FIGS. 21 to 23. Young parasites attached to adjacent dehemoglobinized corpuscular mounds. In Fig. 21 pigment granules may be seen at o. Fig. 23 shows two parasites encircling one of the decolorized mounds. The two parasites in Fig. 22 appear to be in a similar stage of development to those seen in Fig. 21, and to have done as much damage to the infected corpuscle, yet pigment granules are seen only in connection with the parasites in Fig. 21. These figures correspond to Figs. 121, 119, and 122.

FIG. 24. Four parasites are attached to this corpuscle. There are two decolorized hemoglobin mounds, each encircled by two parasites.

FIG. 25. Five parasites are attached to the corpuscle, two of them to the peripheral mound at x. Note the variation in size of the chromatin masses.

FIG. 26. Five parasites are attached to the corpuscle. Note the variation in the development of these parasites.

FIG. 27. Six parasites are attached to the corpuscle, two to each hemoglobin mound. Two of the mounds are decolorized; the parasites attached to the third and central mound have attached themselves more recently than the other parasites. This figure corresponds to Fig. 124.

FIG. 28. Six parasites are attached to the corpuscle, two to each hemoglobin mound. These mounds are all decolorized and a pigment granule is seen at o.

FIG. 29. Seven parasites are attached to the corpuscle, two to each decolorized mound and one to the mound which has not yet been decolorized. This figure corresponds to Fig. 125.

FIG. 30. Four parasites are attached to the corpuscle, two to one mound, and two to separate and adjacent mounds. This figure corresponds to Fig. 123.

FIGS. 31 to 35. Examples of two parasites attached to one surface hemoglobin mound. These parasites give the appearance of developing crescents much more than do the parasites attached to adjoining mounds; but they are only accidentally so attached, and not conjugating. Fig. 35 was taken from a tertian infection. Note the relatively larger size of the chromatin masses of the tertian parasites. Three free parasites may be seen in Fig. 34. Figs. 32 and 34 correspond to Figs. 126 and 127.

PLATE 37.

ÆSTIVO-AUTUMNAL PARASITES.

Magnification, $\times 1,840$.

FIG. 36. Two young parasites freed from a corpuscle which they have destroyed. This conclusion was reached because of the size of the parasites and the presence of a pigment granule at o.

FIGS. 37 and 38. Examples of young parasites encircling peripheral hemoglobin mounds. The parasites in Fig. 38 are older than those in Fig. 37.

FIG. 39. Two parasites encircling with their pseudopodia a peripheral hemoglobin mound. These parasites are larger than those seen in Figs. 37 and 38. Pigment granules may be seen at o. This figure corresponds to Fig. 107.

FIG. 40. Three very young parasites encircling one large peripheral hemoglobin mound. A pigment granule may be seen at o. This is one of the largest peripheral mounds that I have seen. This figure corresponds to Fig. 108.

FIG. 41. At o two young parasites encircle a peripheral hemoglobin mound, while at x two parasites, in a later stage of development, encircle one surface hemoglobin mound. This figure corresponds to Fig. 106.

FIGS. 42 to 46. Examples of two parasites attached to one surface hemoglobin mound. These parasites have developed a thickening of one segment. A pigment granule is seen in connection with one of the parasites in Fig. 46. In Figs. 42 and 43 the parasites have destroyed the corpuscle to which they were attached and are free in the position they occupied when attached to the corpuscle.

FIG. 47. There are four parasites attached to this corpuscle; three to one mound, and one to an adjoining mound. Note the small size of one of the chromatin masses. A pigment granule is seen at o.

FIG. 48. Two parasites encircling one surface hemoglobin mound. A pigment granule is seen at o. This figure corresponds to Fig. 109.

FIG. 49. Three parasites attached to one surface hemoglobin mound. Note the variation in the size of the chromatin masses.

FIG. 50. Two parasites encircling one hemoglobin mound; the body of one of

the parasites rests on the periphery of the corpuscle. A pigment granule is seen at o.

FIGS. 51 and 52. Examples of young parasites encircling one surface hemoglobin mound. These parasites are in similar stages of development; the hemoglobin mound is larger in Fig. 52 than in Fig. 51.

FIGS. 53 and 54. Examples of two parasites in varying stages of development attached to one surface hemoglobin mound. Fig. 53 corresponds to Fig. 110.

FIGS. 55 to 61. Examples of two parasites encircling, in various positions in relation to each other, one surface hemoglobin mound. Fig. 59 shows four parasites, two attached to each surface hemoglobin mound. Figs. 55 and 56 correspond to Figs. 128 and 129.

FIGS. 62 to 67. Examples of three parasites encircling one hemoglobin mound. Note the variation in the size of the chromatin masses in Figs. 62 and 65. In Fig. 65 the hemoglobin mound to which the parasites are attached is easily seen at the periphery of the corpuscle. Fig. 64 corresponds to Fig. 111.

FIGS. 68 and 69. Examples of four parasites encircling one surface hemoglobin mound. Note the variation in size of the chromatin masses. These figures correspond to Figs. 114 and 115.

FIG. 70. Five young parasites encircling one surface hemoglobin mound. There is quite a variation in the size of the chromatin masses. This figure corresponds to Fig. 116.

PLATE 38.

ÆSTIVO-AUTUMNAL PARASITES.

Magnification, $\times 1,840$.

FIGS. 71 to 75. Instances of multiple infection of red corpuscles by young parasites (four to seven). Each chromatin mass corresponds to one or two parasites. In Fig. 71 the parasites are very young and have attached themselves to the periphery of the corpuscle, two at the top, two at the right, and two at the left. Fig. 72 shows four parasites, Fig. 73, seven parasites, and Figs. 74 and 75, five parasites. In Figs. 72 to 75 note the variation in size of the chromatin masses. Figs. 73 and 74 correspond to Figs. 118 and 117.

FIG. 76. A crescent and a young parasite attached to the same red corpuscle. This figure corresponds to Fig. 130.

FIGS. 77 to 98 and 101 to 105. Examples of two crescents attached to one red corpuscle. In Figs. 83, 86 to 88, 90 to 93, 96, and 101, the mounds of hemoglobin substance to which the crescents have attached themselves are well shown. In Fig. 93 the hemoglobin mounds have been dehemoglobinized by the parasites. In Fig. 94 a pseudopodium arising from the cytoplasm of the parasite is shown in the form of a large loop.

FIGS. 81, 82, 83, 84, 86, 87, 88, 90, 101, and 105 correspond to Figs. 135, 137, 136, 138, 133, 132, 131, 134, 139, and 140.

FIG. 99. This figure may be variously interpreted. It may be two contracted crescents, it may be a crescent twisted on itself, or it may be a segmenting crescent.

FIG. 100. Three crescents attached to one corpuscle. This showed better in the stained specimen than it does in the photograph.

PLATE 39.

ÆSTIVO-AUTUMNAL PARASITES.

Magnification, $\times 1,840$.

FIG. 106. Four young æstivo-autumnal parasites attached to a corpuscle; two encircle a peripheral hemoglobin mound, and two, in a more advanced stage of development, encircle a surface hemoglobin mound. This figure corresponds to Fig. 41.

FIG. 107. Two young æstivo-autumnal parasites, with abundant cytoplasm, encircling a peripheral hemoglobin mound. Two pigment granules are seen in connection with one of the parasites. This figure corresponds to Fig. 39.

FIG. 108. Three very small æstivo-autumnal parasites encircling a very large peripheral hemoglobin mound. This figure corresponds to Fig. 40.

FIG. 109. Two young æstivo-autumnal parasites, in similar stages of development, encircling one surface hemoglobin mound. A pigment granule is seen in connection with one of the parasites. It is easily seen that these are individual parasites. This figure corresponds to Fig. 48.

FIG. 110. Two young æstivo-autumnal parasites, in varying stages of development, encircling one surface hemoglobin mound. This figure corresponds to Fig. 53.

FIG. 111. Three young æstivo-autumnal parasites, in similar stages of development, encircling one surface hemoglobin mound. This figure corresponds to Fig. 64.

FIG. 112. Four very young æstivo-autumnal parasites encircling one surface hemoglobin mound. Note the variation in size of the chromatin masses. This figure corresponds to Fig. 8.

FIG. 113. Five parasites are attached to this corpuscle. Four of them encircle one surface hemoglobin mound; a pigment granule is seen in connection with one of these. This figure corresponds to Fig. 10.

FIG. 114. Four young æstivo-autumnal parasites encircling one surface hemoglobin mound. These parasites are in varying stages of development. Note the variation in size of the chromatin masses. This figure corresponds to Fig. 68.

FIG. 115. Four young æstivo-autumnal parasites, in varying stages of development, encircling one surface hemoglobin mound. One of the chromatin masses is slightly smaller than the others. This figure corresponds to Fig. 69.

FIG. 116. Five young æstivo-autumnal parasites, in varying stages of development, attached to one surface hemoglobin mound. Note that one of the chromatin masses is distorted. This figure corresponds to Fig. 70.

FIG. 117. Five young æstivo-autumnal parasites attached to surface hemoglobin mounds. Note that these parasites are not all in similar stages of development. This figure corresponds to Fig. 74.

FIG. 118. Seven young æstivo-autumnal parasites attached to surface hemoglobin mounds. Note the small size of one of these parasites. This figure corresponds to Fig. 73.

FIG. 119. Two young æstivo-autumnal parasites attached to adjacent decolorized hemoglobin mounds. This figure corresponds to Fig. 22.

FIG. 120. Two young æstivo-autumnal parasites encircling two decolorized hemoglobin mounds. A granule of pigment may be seen in connection with one of these parasites. A portion of the cytoplasm of one parasite is seen to overlies a portion of the cytoplasm of the parasite attached to the adjacent hemoglobin mound. This figure corresponds to Fig. 12.

FIG. 121. Two young æstivo-autumnal parasites attached to adjoining hemoglobin mounds. The mounds are decolorized and a pigment granule is seen in connection with each parasite. This figure corresponds to Fig. 21.

FIG. 122. Three young æstivo-autumnal parasites are attached to this corpuscle. Two encircle the decolorized hemoglobin mound at the right (a pigment granule is seen in connection with them), and one encircles the decolorized mound at the left. This figure corresponds to Fig. 23.

FIG. 123. Four young æstivo-autumnal parasites are attached to this corpuscle. Two of them encircle one decolorized hemoglobin mound. This figure corresponds to Fig. 30.

FIG. 124. Six young æstivo-autumnal parasites are attached to this corpuscle, two attached to each hemoglobin mound. Two of the hemoglobin mounds have been decolorized by the action of the parasites. This figure corresponds to Fig. 27.

FIG. 125. Seven young æstivo-autumnal parasites are attached to this corpuscle. Two parasites encircle each of the three decolorized hemoglobin mounds; pigment granules are seen in connection with these parasites. This figure corresponds to Fig. 29.

FIG. 126. Two young æstivo-autumnal parasites attached to one surface hemoglobin mound. This figure corresponds to Fig. 32.

FIG. 127. Two young æstivo-autumnal parasites attached to one surface hemoglobin mound. To the right may be seen three young parasites free. This figure corresponds to Fig. 34.

FIGS. 128 and 129. Examples of two young æstivo-autumnal parasites attached to one surface hemoglobin mound. One of the two parasites in Fig. 129 rests on the periphery of the corpuscle. These figures correspond to Figs. 55 and 56.

FIG. 130. A young æstivo-autumnal parasite and a crescent attached to the same red corpuscle. This figure corresponds to Fig. 76.

FIGS. 131 to 140. Examples of two crescents on one corpuscle. In Figs. 131 to 136 and 139, the mounds of hemoglobin substance to which the crescents are attached are well shown. These figures correspond to Figs. 88, 87, 86, 90, 81, 83, 82, 84, 101, and 105.



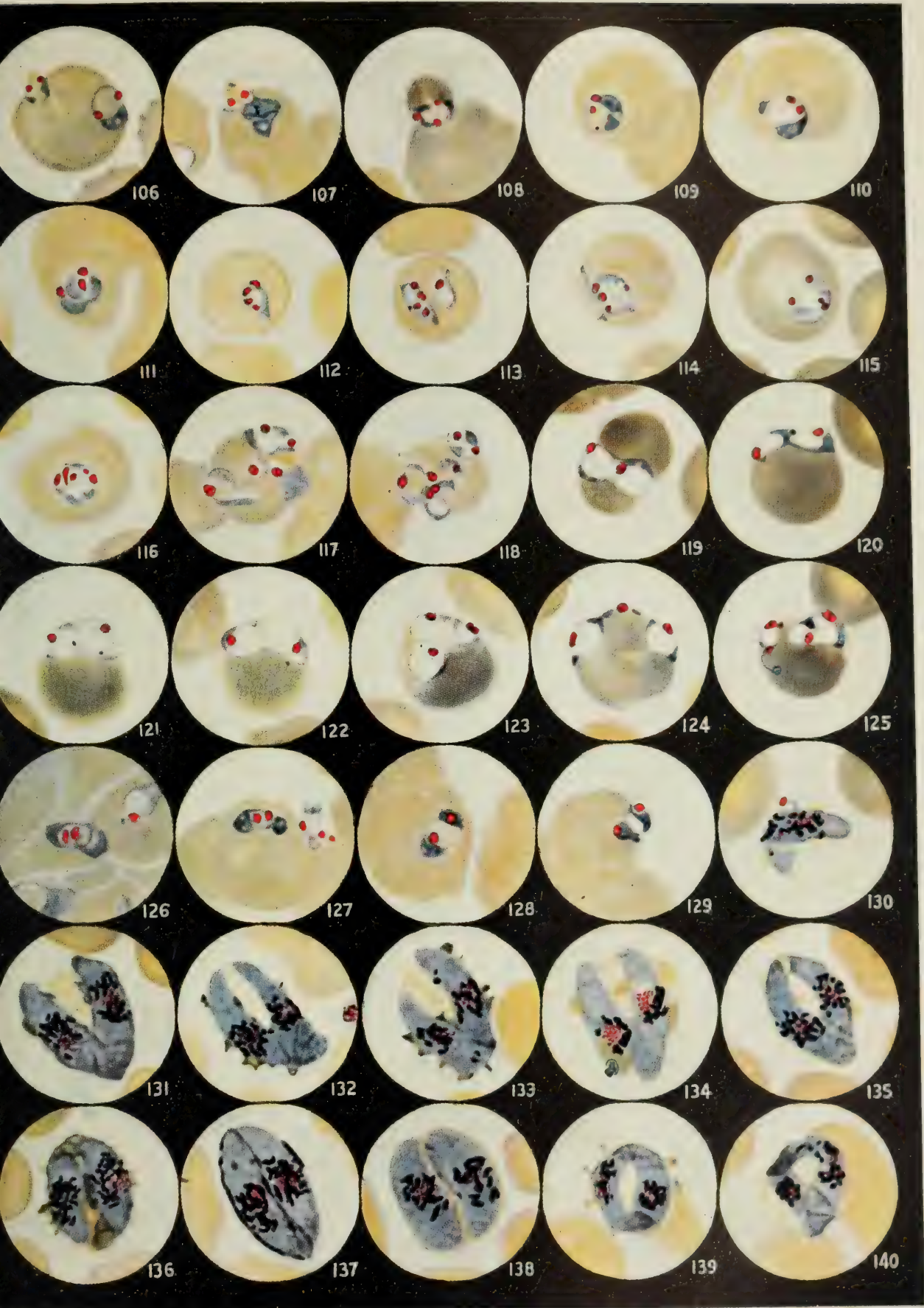
(Lawson: Multiple infection of red corpuscles.)



(Lawson: Multiple infection of red corpuscles.)



(Lawson: Multiple infection of red corpuscles.)



(Lawson: Multiple infection of red corpuscles.)

THE LYMPHOCYTE IN NATURAL AND INDUCED RESISTANCE TO TRANSPLANTED CANCER.

III. THE EFFECT OF X-RAYS ON ARTIFICIALLY INDUCED IMMUNITY.*

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In previous communications it has been shown (a) that the normal chick embryo lacks the ability to destroy heteroplastic tissue grafts;¹ (b) that this lack of resistance disappears between the 19th and 21st days of incubation, and it is significant that the spleen develops at about this time;² (c) that the lack of resistance, seen during the early days of the incubation period, is replaced by a degree of resistance comparable with that observed in the adult animal if the embryo is supplied with a small bit of adult lymphoid tissue;³ (d) that the natural resistance of adult animals to heteroplastic tissue grafts can be destroyed by the x-rays in proper dosage;⁴ and (e) that the disappearance of tissue grafts in resistant animals is associated with an accumulation of lymphocytes about them.⁵

It is known⁶ that mice injected subcutaneously with homologous living tissue cells, after an interval of about 10 days, are potentially immune to tissues subsequently inoculated from the same species; namely, mouse cancers. This potential immunity can be readily destroyed by exposing the animals to suitable doses of the x-rays in the interval between the immunizing dose and the cancer inocula-

* A preliminary announcement of these experiments was made by Murphy and Taylor before the American Association for Cancer Research, New York, April 5, 1917, and was published in abstract form in the proceedings of this meeting (*J. Cancer Research*, 1917, ii, 504). Since this report a paper has appeared by Mottram and Russ, repeating and confirming these observations (Mottram, J. C., and Russ, S., *Proc. Roy. Soc. London, Series B*, 1917-18, xc, 1).

This investigation was carried out by means of funds from the Rutherford Donation.

¹ Murphy, Jas. B., *J. Am. Med. Assn.*, 1912, lix, 874.

² Murphy, Jas. B., *J. Exp. Med.*, 1913, xvii, 482.

³ Murphy, Jas. B., *J. Exp. Med.*, 1914, xix, 513.

⁴ Murphy, Jas. B., *J. Am. Med. Assn.*, 1914, lxii, 1459.

⁵ For review of literature see Da Fano, C., *Z. Immunitätsforsch., Orig.*, 1910, v, 1.

⁶ Imperial Cancer Research Fund, *Brit. Med. J.*, 1906, ii, 209.

tion. This finding was interpreted as being due to interference with the lymphoid blood crisis which has been shown to follow the tumor inoculation⁷ and which has been thought to influence the subsequent resistance.

It is established that the potential immunity to cancer resulting from an homologous tissue injection is of the nature of a non-specific reaction, the resistance produced being directed toward a great variety of cancers and sarcomas as well as toward homologous normal tissues. Injection with one of the transplantable mouse carcinomata renders the animal resistant to that tumor alone and thus the reaction becomes specific. It seemed possible that while the lymphocyte might be a potent factor in bringing about the potential immunity, which is non-specific, after the resistance becomes specifically directed against a particular tumor, this cell might no longer play a part in the maintenance of the immunity. To test this point the following experiments were carried out.

Method.

Mice were immunized by an injection of homologous defibrinated blood beneath the skin of the back. 10 days later a bit of tumor (Bashford Adenocarcinoma No. 63) was inoculated into the left groin of each animal. A number of non-immunized mice were inoculated at the same time with the tumor in order to control its virulence. After the animals had been observed for a period of 3 weeks, the immune animals were divided and one group was subjected to small repeated doses of x-rays, the other being set aside for controls. A week later both groups were reinoculated in the right groin with the same tumor strain, the virulence of the strain being determined by simultaneous inoculation into normal mice. The x-ray dosage used in these experiments was one which previous experiments⁸ had shown to be adequate to destroy the major portion of the lymphoid tissue without appearing to affect the general health of the animal.

Experiment 1.—Forty-nine white mice of the same approximate age, size, and weight, obtained from one source, at the same time, were injected beneath the skin of the back with 0.2 cc. of defibrinated mouse blood. 10 days later there was

⁷ Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204.

⁸ Murphy, Jas. B., and Ellis, A. W. M., *J. Exp. Med.*, 1914, xx, 397. Taylor, H. D., and Murphy, Jas. B., *J. Exp. Med.*, 1917, xxv, 609.

inoculated into the left groin of each a bit of Bashford Adenocarcinoma No. 63. At the end of the 3 weeks interval following inoculation, forty animals, or 81.6 per cent, were found to be immune. Of ten normal animals inoculated simultaneously as controls, nine, or 90 per cent, developed tumors. It is evident, therefore, that the tumor is freely transplantable to normal animals and that the resistance present in those previously injected with mouse blood represents an induced immunity.

After a period of 3 weeks following the first inoculation with the tumor, the forty immune mice were divided into two groups. The first, comprising nineteen animals, remained untreated, while the second, comprising twenty-one animals, was exposed to the x-rays for 7 successive days. The dose of x-rays given at each exposure was as follows: a $2\frac{1}{4}$ inch spark-gap, 10 milliamperes, with a distance of 12 inches between the target of the Coolidge tube and the nearest point on the backs of the mice, time of exposure 2 minutes. After the seven exposures both groups of immune mice and eleven normal controls as well were inoculated with a later generation of the Bashford tumor in the right groin, with the results shown in Table I.

TABLE I.

Group.	Treatment.	Per cent of takes.
I	Immunized.	21.0
II	“ and x-rayed.	52.4
III	Controls.	90.9

Of the animals artificially immunized against, inoculated with, and proven immune to a transplantable carcinoma, those which received no x-ray treatment before a second inoculation with the same tumor still showed a high degree of immunity, as only four of the nineteen, or 21 per cent, developed tumors. The second group of mice, which was x-rayed before the second tumor inoculation, showed a much smaller degree of immunity, eleven mice, or 52.4 per cent, yielding tumors. Of the controls, ten, or 90.9 per cent, developed tumors.

Experiment 2.—Fifty-two mice were immunized and inoculated with the mouse carcinoma in the manner indicated in Experiment 1; of these, fifty-one, or 98 per cent, proved to be immune. It happened that nine of the fifteen control mice developed tumors, so that 40 per cent showed natural immunity. During the 3rd week following the initial tumor inoculation twenty-five of the animals received seven daily x-ray treatments with a Coolidge tube, the following factors being used at each exposure: spark-gap $2\frac{1}{4}$ inches, milliamperes 10, distance from the target to the backs of the mice 12 inches, and the time of exposure 2 minutes.

Twenty-five of the immune animals received no x-ray treatment. The two groups, fifty mice in all, together with eighteen control animals, were then inoculated in the right groin with a bit of a later generation of the Bashford tumor used in the primary inoculation. The results are shown in Text-fig. 1 and Table II.

TABLE II.

Group.	Treatment.	Per cent of takes.
I	Immunized.	12.0
II	“ and x-rayed.	64.0
III	Controls.	94.4

Of the immune mice not treated with x-rays, only three, or 12.0 per cent, developed tumors. Of the immune animals x-rayed before the second inoculation, sixteen, or 64 per cent, developed tumors. There was tumor growth in seventeen, or 94.4 per cent, of the eighteen control animals.

TABLE III.

Group.	Treatment.	No. of mice.	Before x-ray treatment.	After x-ray treatment.
			Per cent of takes.	Per cent of takes.
I	Immunized.	19*	10.5	73.3
II	Controls.	10†	90.0	90.0

* Fifteen surviving at second inoculation.

† Ten mice as controls for each inoculation of Group I.

Experiment 3.—As shown in Table III, nineteen mice were inoculated in the left groin with a bit of the Bashford tumor after having received, 10 days before the tumor inoculation, 0.2 cc. of defibrinated mouse blood. Two mice, or 10.5 per cent, developed tumors. Six doses of x-rays with the Coolidge tube were then administered on successive days, the daily dose depending on the following factors: spark-gap $2\frac{1}{4}$ inches, milliamperes 10, distance from target to mouse 12 inches, and time of exposure 1 minute. The fifteen mice which survived the period were then inoculated in the right groin with a later generation of the Bashford tumor and eleven, or 73.3 per cent, developed tumors. There was tumor growth in nine, or 90 per cent, of the ten animals used as controls to the first inoculation and the same percentage of growth in the same number of controls to the second inoculation.

Experiment 4.—Mice immunized and inoculated as indicated in the previous experiments were divided into two lots. After inoculation with the Bashford

tumor the first lot, consisting of nine mice, showed tumors in three, or 33.3 per cent. The second group of eight immune animals was given a series of x-ray treatments identical with those of Experiment 2, and 50 per cent developed tumors. There were 66.6 per cent of takes in the nine control animals (Table IV).

TABLE IV.

Group.	Treatment.	Per cent of takes.
I	Immunized.	33.3
II	“ and x-rayed.	50.0
III	Controls.	66.6

Experiment 5.—Twenty-seven animals immunized as in the previous experiments were inoculated in the left groin with the Bashford tumor and to control further the immunity which was evident at this time were reinoculated with the same tumor. All proved to be immune after this rigid test. Seventeen of these immune mice gave 29.4 per cent of takes after a third inoculation with the same tumor to control the ten remaining ones which were also inoculated with this tumor after x-ray treatments on 3 successive days in the same manner as that employed in Experiments 1, 2, and 3, except that the time of exposure was 3 minutes for each of four exposures. Five of the second group, or 50 per cent, developed tumors. Four, or 40 per cent, of the ten control animals developed tumors (Table V).

TABLE V.

Group.	Treatment.	Per cent of takes.
I	Immunized.	29.4
II	“ and x-rayed.	50.0
III	Controls.	40.0

Experiment 6.—Twenty mice were immunized and proved immune in the manner described in the preceding experiments, divided into two groups, and one group was given x-ray treatments with the Coolidge tube as follows: $2\frac{1}{4}$ inch spark-gap, 10 milliamperes, 12 inch distance. Five of the daily exposures were of 2 minutes each and one for 5 minutes, the exposures being made during the 3rd week following the initial tumor inoculation. Of the seven animals so treated, 85.7 per cent developed tumors when given a second inoculation in the right groin with the Bashford tumor. A control group of thirteen untreated immune animals from the same lot of mice, inoculated at the same time, showed tumor growth in only 38.8 per cent. 60 per cent of the ten inoculated normal mice developed tumors. The results of the experiment are given in Table VI.

TABLE VI.

Group.	Treatment.	Per cent of takes.
I	Immunized.	38.8
II	“ and x-rayed.	85.7
III	Controls.	60.0

Experiment 7.—Seven mice inoculated after being immunized in the usual way gave 28.2 per cent of takes. Inoculated a second time, only 14.2 per cent grew tumors. Of seven other mice, from the same group, 14.2 per cent developed tumors, after an inoculation with the Bashford tumor before treatment. After five daily exposures to the x-rays generated by a Coolidge tube there were 71.3 per cent of takes in this second group. The x-ray factors were the same as those used in Experiment 1. Control mice inoculated at the same time, with the same tumor, showed 71.3 per cent of tumors. The results of the experiment are given in Table VII.

TABLE VII.

Group.	Treatment.	First inoculation. Per cent of takes.	Second inoculation. Per cent of takes.
I	Immunized.	28.2	14.2
II	“ and x-rayed.*	14.2*	71.3*
III	Controls.	71.3	—

* X-ray exposures after first and before second inoculation.

Experiment 8.—Twenty-eight mice were immunized, each with 0.2 cc. of defibrinated mouse blood, and divided into two groups. The first group of nine gave 11.1 per cent tumors after the first inoculation into the left groin and 37.5 per cent after the second inoculation into the right groin. They had no x-ray treatment. Of the second group of nine mice, x-rayed before the first inoculation, 55.5 per cent developed tumors. This group was again x-rayed and reinoculated into the right groin. 80 per cent developed tumors. A third group of immune animals was inoculated first in the left groin with the tumor, of which 20 per cent developed tumors. They were x-rayed, and upon reinoculation in the right groin 57.1 per cent of tumors resulted. The x-ray dosage was the same in both series of treatments given the second group of mice, as well as in the series of treatments which the third group received. The x-ray factors were identical with those of Experiment 1. The control mice gave 80 per cent of tumors. The results are given in Table VIII.

TABLE VIII.

Group.	Treatment.	First inoculation. Per cent of takes.	Second inoculation. Per cent of takes.
I	Immunized. No x-ray treatment.	11.1	37.5*
II	Immunized. X-rayed before first and second inoculations.	55.5	80.0†
III	Immunized. X-rayed before second inoculation.	20.0	57.1‡
IV	Controls.	80.0	—

* Only eight living at time of second inoculation.

† Only five living at time of second inoculation.

‡ Only seven living at time of second inoculation.

DISCUSSION.

The experiments described in this paper indicate anew that the lymphocytes are a potent factor in the immunity to cancer which has been studied in the mouse. Taken with other indications the evidence is growing in importance and conclusiveness of the part played by the lymphocyte in bringing about and in maintaining that condition. The main points of evidence now adducible are: (1) the accumulation of lymphocytes about a transplanted cancer graft in an immunized animal; (2) the rise in number in the circulating lymphocytes during the development of the immune state, irrespective of whether the type of immunity induced is artificial or natural; (3) the setting aside of the potential immunity by the x-rays where the dosage employed is sufficient to destroy a large part of the circulating lymphocytes; and finally, (4) as shown by the present experiments, the abolition of the potential immunity for a special tumor strain by means of the lymphocyte-destroying power of the x-rays. These specific points are further supported by the observations of Leo Loeb⁹ on the part played by the lymphocyte in respect to homoplastic grafts of normal tissue, and by those of Murphy³ on heteroplastic tissue grafts.

⁹ Loeb, L., *J. Med. Research*, 1917, xxxvii, 229.

SUMMARY.

Mice artificially immunized against a transplantable carcinoma, inoculated, and proved immune, may be again rendered susceptible to the same tumor by exposure to the x-rays.

The immune animals which have not been treated with the x-rays preserve, to a large degree, their resistance to a second inoculation of the tumor in question.

THE PASSAGE OF NEUTRALIZING SUBSTANCE FROM THE BLOOD INTO THE CEREBROSPINAL FLUID IN ACTIVELY IMMUNIZED MONKEYS.

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Previous experiments¹ have shown that in passively immunized monkeys neutralizing antibodies for the poliomyelitic virus can be made to pass from the blood into the cerebrospinal fluid by merely increasing the permeability of the meningeal-choroidal complex, through an aseptic inflammation induced by means of normal horse serum. The passive immunization is effected through the injection into normal monkeys of the blood serum of monkeys which have survived an attack of experimental poliomyelitis and which subsequently have had the immunity reinforced by subcutaneous injection of active virus (suspensions of spinal cord and brain of recently paralyzed monkeys preserved in glycerol). The order of the experiment was as follows: An aseptic meningitis was induced in normal monkeys by an intraspinal injection of 2 cc. of normal horse serum. The next morning, or about 16 hours later, about 10 cc. of the immune serum were injected intravenously. At intervals of 6, 9, and 24 hours fluid was withdrawn by lumbar puncture and employed for neutralization tests. The 6 and 9 hour specimens were combined so that the tests were actually made with samples of cerebrospinal fluid taken 6 and 9 hours and 24 hours after the horse serum was introduced. The control tests were made with normal horse serum. The procedure, as far as the actual neutralization is concerned, was identical with that of the present experiments. The results show that normal horse serum is devoid of neutralizing power for the virus; that at the expiration of 6 to 9 hours sufficient amount of the antibodies introduced into the blood had already passed into the cerebrospinal fluid to effect neutralization of the virus; and that the fluid withdrawn after 24 hours might no longer neutralize the virus perfectly.

These experiments were regarded as having a certain significance in respect to the specific therapy of poliomyelitis. Flexner and Lewis² and Flexner and Amoss³ had already shown that the introduction of immune monkey or immune

¹ Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1917, xxv, 499.

² Flexner, S., and Lewis, P. A., *J. Am. Med. Assn.*, 1910, liv, 1780; lv, 662.

³ Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1914, xx, 249; 1917, xxv, 525.

human serum into the meninges of monkeys prevented experimental infection with active poliomyelitic virus. Netter and his associates⁴ first applied the principles of this observation to the treatment of cases of poliomyelitis in man. Later others, notably Amoss and Chesney,⁵ reported series of cases treated in this manner with immune (convalescent) human serum. The promising results obtained led to the employment by others of normal sera. Thus Sophian⁶ injected normal horse serum and Zingher⁷ normal human serum intraspinaly in cases of acute poliomyelitis. Their results were not definite, and yet they have been regarded as favorable in some instances.

Since normal serum exhibits no neutralizing action on the poliomyelitic virus *in vitro*, the possibility exists that the normal sera may serve merely to divert the neutralizing substances from the blood into the meninges by increasing the permeability of the meningeal-choroidal complex. It is now known that these bodies are detectable in the circulating blood of man in some instances as early as the 3rd day⁸ of an attack of poliomyelitis. Hence the possibility exists that cases reacting favorably to intraspinal injections of horse serum may have endured long enough at the time of injection to be benefited by the diversion of immune bodies indicated.

Under these circumstances the diversion would take place not in a passively but in an actively immune person. It seemed desirable, therefore, to make actual tests upon actively immune monkeys. A number of animals which had recovered from attacks of experimental poliomyelitis were available. They had all subsequently been injected with virus suspensions to increase (reinforce) their immunity.

EXPERIMENTAL.

The specific experiments made to ascertain the presence of neutralizing substances in the cerebrospinal fluid were preceded by a series of

⁴ Netter, A., Gendron, A., and Touraine, *Compt. rend. Soc. biol.*, 1911, lxx, 625. Netter, A., *Bull. Acad. méd.*, 1915, lxxiv, series 3, 403. Netter, A., and Salanier, M., *Bull. et mém. Soc. méd. hôp. Paris*, 1916, xl, series 3, 299.

⁵ Amoss, H. L., and Chesney, A. M., *J. Exp. Med.*, 1917, xxv, 581.

⁶ Sophian, A., *J. Am. Med. Assn.*, 1916, lxxvii, 426.

⁷ Zingher, A., *Dept. Health, City of New York, Reprint Series, No. 54*, 1917.

⁸ Kling, C., and Levaditi, C., *Études sur la poliomyélite aiguë épidémique*, Paris, 1913, 114.

tests to determine whether active complement is actually required to accomplish the destruction of the poliomyelitic virus *in vitro*. Diverse opinions on this point prevail.⁹ Our previous experiments had led us to believe fresh complement not essential. On the other hand, irregularities not always readily explained sometimes arise in the course of the neutralization tests. As sometimes fresh and sometimes stored sera have been employed, the lack of uniformity has been attributed to the variation in the complement. As Experiment 1 indicates, inactive sera are perfectly neutralizing. The irregularities probably are to be accounted for rather by the quality of the virus, for a serum which contains in a given volume sufficient antibodies to neutralize a unit of virus of one degree of activity may fail to neutralize this unit of a more intense or active virus. Hence it is imperative to cover all tests on immunity in relation to poliomyelitis with adequate control observations. While the virus once adapted to monkeys by successive passages acquires and retains for a long time a marked virulence, yet quantitative fluctuations occur from time to time which are not predictable and heighten or depress the activity.

Experiment 1. Relation of Neutralization to Active Complement.—For this experiment a glycerolated virus (spinal cord and medulla) was employed. A 5 per cent suspension in isotonic saline solution was prepared, centrifuged, and filtered through a Berkefeld candle. *Macacus rhesus* A was inoculated intracerebrally with 2.6 cc. of a mixture of 2.4 cc. of fresh normal monkey serum and 0.2 cc. of virus filtrate. The mixture of filtrate and serum had been kept at 37°C. for 2 hours and in the refrigerator (4°C.) over night. 6 days after the inoculation the animal was ataxic, showed head tremor, and moved about slowly. Death took place on the 7th day. The autopsy showed the characteristic lesions of poliomyelitis. *Macacus rhesus* B received an intracerebral inoculation of the following: inactivated immune monkey serum 2 cc., inactivated normal serum 0.4 cc., virus filtrate 0.2 cc., mixed and treated as for *Macacus rhesus* A. No symptoms developed. *Macacus rhesus* C was a repetition of *Macacus rhesus* B in which active normal serum replaced the inactive. No symptoms appeared. For Monkey D an inactive human (convalescent) serum was employed in proportion of 2 cc. of serum to 0.2 cc. of virus filtrate. No symptoms developed.

This experiment yielded a clear and definite result. The neutralization of the virus is accomplished directly and without the inter-

⁹ Landsteiner, K., in Kolle, W., and von Wassermann, A., *Handbuch der pathogenen Mikroorganismen*, Jena, 2nd edition, 1913, viii, 449.

vention of fresh complement. This point has an interest in connection with the test with the cerebrospinal fluid which follows, since it shows that the presence of active complement can be disregarded.

Experiment 2. Passage of Neutralizing Substance into the Cerebrospinal Fluid.—This experiment was performed with cerebrospinal fluids withdrawn 6 and 9 hours respectively after the injection of 2 cc. of normal horse serum into the meninges of actively immune monkeys. The two fluids were combined and mixed with the virus filtrate in the manner of Experiment 1. The control tests were made with virus mixed with normal cerebrospinal fluid and with normal horse serum respectively. *Macacus rhesus E* (control) received an intracerebral inoculation of an incubated mixture of 1 cc. of normal cerebrospinal fluid, 0.3 cc. of isotonic saline solution, and 0.2 cc. of filtrate virus. On the 24th day ataxia, right facial paralysis, and weakness of extremities were present. The next day the extremities were paralyzed and death occurred. The autopsy showed lesions of poliomyelitis. *Macacus rhesus F* (control) received an intracerebral inoculation of an incubated mixture of 1 cc. of normal horse serum, 0.3 cc. of isotonic saline solution, and 0.2 cc. of filtrate virus. On the 6th day the extremities were paralyzed and a right facial paralysis existed. The animal was etherized and the autopsy showed marked lesions of poliomyelitis. *Macacus rhesus G* received an intracerebral inoculation of an incubated mixture of 0.2 cc. of virus filtrate and 1 cc. of the combined cerebrospinal fluid withdrawn from an actively immune monkey 6 and 9 hours respectively after the intraspinal injection of normal horse serum. 9 days later ataxia, right facial paralysis, tremor of head, and weak deltoids were present. The next day all the extremities were paralyzed and the animal was etherized. The autopsy showed lesions of poliomyelitis. *Macacus rhesus H* was an exact repetition of Monkey G. On the 12th day ataxia, paralysis of right arm, and weakness of many other muscles were noted. The paralysis extended somewhat and then gradually receded. Recovery with residual paralysis took place.

An immediate interpretation of this result would necessitate the conclusion that passage of neutralizing substances from the blood into the chemically inflamed meninges either did not take place in actively immune monkeys at all, or only inadequately in the period of 6 to 9 hours. Since in the passively immunized monkeys this period sufficed for the passage, some other explanation must be sought. Doubtless it is found in the discrepancy of the experimental procedure in the two series. In the case of the passively immunized monkey, the meningeal inflammation is induced about 16 hours before the immune serum is injected; hence a 9 hour specimen of cere-

brospinal fluid would be withdrawn about 25 hours after the horse serum was injected intraspinally. In the actively immune animals the fluid is withdrawn 9 hours after the horse serum is injected. In the one instance the inflammation is at its height, in the other in process of development when the cerebrospinal fluid is withdrawn. That this is the proper explanation is indicated by Experiment 3.

Experiment 3. Passage of Neutralizing Substance into the Cerebrospinal Fluid.—This experiment is a repetition and extension of Experiment 2. The manner of carrying it out was identical. Normal horse serum was injected intraspinally, and the periods at which the fluid was taken by lumbar puncture were 12, 24, and 48 hours. In the instance of the 48 hour withdrawal, a second injection of horse serum was made at the end of the first 24 hour period, in order to maintain the inflammation at a high level. Inoculations of monkeys with the cerebrospinal fluid and virus mixtures were made in duplicate, in order to cover any unforeseen variation in the results. *Macacus rhesus* I (control) received an intracerebral injection of an incubated mixture of 2 cc. of normal horse serum and 0.2 cc. of virus filtrate. On the 4th day the animal was excited; on the 5th, the extremities were all paralyzed and death resulted. The autopsy disclosed marked lesions of poliomyelitis. *Macacus rhesus* J and J' each received an intracerebral inoculation of an incubated mixture of 0.2 cc. of virus filtrate and 1 cc. of cerebrospinal fluid withdrawn from an actively immune monkey 12 hours after an intraspinal injection of normal horse serum. No symptoms developed. *Macacus rhesus* K and K' received similar injections of mixtures containing 24 hour specimens of cerebrospinal fluid and 0.2 cc. of virus filtrate. No symptoms developed. *Macacus rhesus* L and L' received identical injections of mixtures containing 48 hour specimens of cerebrospinal fluid and 0.2 cc. of virus filtrate. No symptoms appeared.

This experiment is conclusive. It shows that beginning 12 hours after the normal horse serum is injected into the meninges of actively immune monkeys, and at a period when the inflammation induced may be regarded as marked, readily measurable quantities of the neutralizing antibodies were poured into the cerebrospinal fluid. This passage continues for 48 hours at least, that is considerably longer than in the passively immunized animals, as might have been predicted. Probably the passage would continue as long as the permeability of the meningeal-choroidal complex persisted. The results of this experiment indicate also that the explanation offered for the failure of Experiment 2 is probably the correct one.

The results of the experiments make clearer the manner in which recovery from poliomyelitis may be supposed to be brought about, and throw light on the probable value of a serum therapy. Immune bodies do not pass normally from the blood to the cerebrospinal fluid, which is, as it were, the lymph of the central nervous system (Mott¹⁰). In poliomyelitis, however, the entire vascular system of the meninges and affected portions of the solid nervous organs, as well as the structures of the choroid plexus, are often so severely injured as to be rendered readily permeable to the protein of the plasma and hence to immune bodies contained in it. The latter should therefore begin to appear in the cerebrospinal fluid just as soon as they begin to accumulate in the blood, and from that fluid permeate to the interior of the central nervous organs. From the moment this transfer of antibodies begins, the neutralization of the virus present in the nervous tissues would also begin; and gradually or quickly in the non-fatal cases an arrest of multiplication of the virus would be effected. That the cessation of the extension of the paralysis occurs very quickly in some cases is a matter of common observation. The presence of the neutralizing antibodies seems to be wholly determined by an excessive permeability of the blood vessels of the nervous system, for once their integrity is restored and the cerebrospinal fluid has returned approximately to normal composition, neutralizing antibodies can no longer be detected there.¹¹ By the time the acute process is at an end, the infection has run its course. There remains to be accomplished merely the restoration, as far as may be, of the organic and functional integrity of the injured structures.

CONCLUSIONS.

For the neutralization of the virus of poliomyelitis by antibodies, active complement is not required.

In carrying out immunity tests it is imperative to choose a virus of established grade of virulence and to make adequate control observations.

¹⁰ Mott, F. W., *Lancet*, 1910, ii, 79.

¹¹ Flexner, S., Clark, P. F., and Amoss, H. L., *J. Exp. Med.*, 1914, xix, 205.

The neutralizing substances pass from the blood of actively immune monkeys into the cerebrospinal fluid when the permeability of the meningeal-choroidal complex is increased by an aseptic inflammation such as that induced by an intraspinal injection of horse serum.

The immunity bodies in effective neutralizing quantities can be detected in the cerebrospinal fluid as early as 12 hours and as late as 48 hours after the intraspinal injection of horse serum. Doubtless the passage continues as long as the inflammation persists.

This ability of the neutralizing substances to pass from the blood into the cerebrospinal fluid under conditions of inflammation doubtless plays an important part in arresting the multiplication of the virus on which the cessation and restoration of the poliomyelitic processes depend. The widespread involvement in the inflammatory conditions of the meninges, choroid plexus, and substance of the nervous organs, accompanied by severe lesions of the blood vessels in the last structures especially, opens the way widely for the passage of antibodies into the cerebrospinal fluid, whence all parts of the nervous tissues are reached, and also, probably, for direct transudation into the affected parts of the spinal cord and brain. The neutralization of the virus on which the continuance of the active pathological process depends is thus readily accomplished.

Under these circumstances the use of an alien specific immune serum to anticipate the action of the individual's own immunity products appears logical, while the employment of normal serum has no basis in experiment and would seem not to offer any therapeutic advantage whatever.

A HOMOHEMOLYTIC SYSTEM FOR THE SERUM DIAGNOSIS OF SYPHILIS.

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There is little doubt that the elimination of the use of guinea pig complement from the serodiagnostic procedure is a great advance towards the simplification of this important reaction, for that element has to be used while it is perfectly fresh, necessitating the maintenance of guinea pigs in the laboratory. Attempts to preserve the activity of the complement, either by means of desiccation¹ or hypertonization,² have been only partially successful, and it deteriorates in a comparatively short time. The best preservative for guinea pig complement is that recommended by Rhamy,³ who found that sodium acetate in appropriate concentration keeps the complement active for several weeks.

Some years ago Hecht⁴ proposed the utilization of the natural anti-sheep hemolysin and complement in the fresh serum of patients, but the difficulty in this method lies in the fact that in some sera there is no natural hemolysin or too little to produce hemolysis, even in the control tubes without the antigen. On the other hand, some sera contain a considerable excess of the natural anti-sheep hemolysin. Granting that the insufficiency of the natural anti-sheep hemolysin can be remedied by adding an adequate quantity of a non-syphilitic

¹ Noguchi, H., On the influence of the reaction and of desiccation upon opsonins, *J. Exp. Med.*, 1907, ix, 455; Serum diagnosis of syphilis, and the butyric acid test for syphilis, Philadelphia and London, 1st edition, 1910.

² Austin, F. D., A new method for preserving complement for making the Wassermann or Noguchi blood-test, *J. Am. Med. Assn.*, 1914, lxii, 868.

³ Rhamy, B. W., Preservation of complement. A preliminary report, *J. Am. Med. Assn.*, 1917, lxix, 973.

⁴ Hecht, H., Eine Vereinfachung der Komplementbindungsreaktion bei Syphilis, *Wien. klin. Woch.*, 1909, xxii, 338.

serum containing enough hemolysin, the test still calls for the washed sheep corpuscles. In other words, having eliminated the use of guinea pig serum as complement, we still have to depend upon the sheep corpuscles for an indicator of hemolysis.

In ordinary times there should be no difficulty in obtaining the guinea pig complement or sheep blood corpuscles, and it would be immaterial whether one used the anti-sheep hemolytic system or the anti-human hemolytic method as advocated by the writer⁵ and accepted by many serologists, including those in the United States Army and Navy. Perhaps it may not be amiss to call attention here to the fact that the anti-human hemolytic system of the writer is not limited in its use to unheated sera but applies equally to inactivated sera, the only differences between the examination of fresh and inactivated sera being that only 0.02 cc. of the former is required, instead of 0.08 to 0.1 cc. of inactivated specimens, and that in the case of inactivated sera not only the acetone-insoluble fraction of tissue lipoids, but also any properly titrated alcoholic extracts may be used as the antigen.⁶ With fresh sera emphasis has been placed on the fact that the crude alcoholic extracts or those alcoholic extracts to which cholesterol has been added are to be avoided, as in this combination there is apt to occur a non-specific proteotropic complement fixation.⁷ It is therefore essential that only the acetone-insoluble fraction of tissue lipoids should be used in combination with fresh sera. With these points in view there should be no confusion as to the extent of applicability of the test to fresh and inactivated sera.⁶

There is another possibility, namely that by eliminating the use of the guinea pig complement from the anti-human hemolytic system the technique and the material for the serodiagnosis of syphilis can be greatly simplified. That this is the case is shown below. Efforts to introduce similar methods have already been made by Tscher-

⁵ Noguchi, A new and simple method for the serum diagnosis of syphilis, *J. Exp. Med.*, 1909, xi, 392.

⁶ Noguchi, Serum diagnosis of syphilis, and luetin reaction, together with the butyric acid test for syphilis, Philadelphia and London, 3rd edition, 1912.

⁷ Noguchi, On non-specific complement fixation, *Proc. Soc. Exp. Biol. and Med.*, 1909-10, vii, 55.

nogubow,⁸ Emery,⁹ Butler and Landon,¹⁰ Myer,¹¹ and Thompson,¹² all employing human complement. A brief review of these methods will be given later.

Principle of the Method.

It is well known that complement is present in every fresh serum and that the quantity may sometimes vary. In the serodiagnosis of syphilis, whether by the anti-sheep system of Wassermann or the anti-human system of the writer, the serum of the guinea pig is chosen because of its richness in complement and also because guinea pig complement is probably more readily fixed¹³ by the antigen-antibody combination than the sera of other animals, such as the horse, rabbit, sheep, pig, etc.^{14, 15} As to the complement in fresh human sera, there are not many data regarding its action upon human corpuscles. It is understood that human sera exert no hemolytic action upon human corpuscles, especially when there is no isohemolysin. But upon the addition of a sufficient quantity of the anti-human hemolytic amboceptor the complement dissolves the human corpuscles just as guinea pig complement does when added to the suspension of human corpuscles and the specific anti-human hemolytic amboceptor.

⁸ Tschernogubow, N. A., Ein vereinfachtes Verfahren der Serumdiagnose be Syphilis, *Deutsch. med. Woch.*, 1909, xxxv, 668.

⁹ Emery, W. d'E., *Clinical bacteriology and hæmatology for practitioners*, Philadelphia, 4th edition, 1912.

¹⁰ Butler, C. S., and Landon, W. F., A technic for the absorption test for syphilis using human complement, *U. S. Nav. Med. Bull.*, 1916, x, 1.

¹¹ Myer, S. B., A complement-fixation test for syphilis using human complement, *U. S. Nav. Med. Bull.*, 1917, xi, 175.

¹² Thompson, L., Complement fixation in syphilis, with a preliminary report of a new technic, *Am. J. Syph.*, 1917, i, 555.

¹³ Noguchi, H., and Bronfenbrenner, J., Variations in the complementary activity and fixability of guinea pig serum, *J. Exp. Med.*, 1911, xiii, 69.

¹⁴ Noguchi, Non-fixation of complement, *Proc. Soc. Exp. Biol. and Med.*, 1909-10, vii, 14.

¹⁵ Noguchi and Bronfenbrenner, The comparative merits of various complements and amboceptors in the serum diagnosis of syphilis, *J. Exp. Med.*, 1911, xiii, 78.

The only difference between the action of the human and the guinea pig complement lies in the fact that the former requires more anti-human hemolytic amboceptor to render it active against the human corpuscles than the latter. The relative lytic values of the human and the guinea pig complement are shown in the following experiments.

*Comparative Complement Values of the Human and the Guinea Pig Sera
Relative to the Anti-Human Hemolytic Amboceptor
and Human Corpuscles.*

0.04 cc. of guinea pig complement was put in each of a number of tubes, together with 1 cc. of a 1 per cent suspension of human corpuscles. Varying quantities of the anti-human hemolytic amboceptor (immunized rabbit) were added to the tubes and the results read after 30 minutes at 37°C. in a water bath thermostat. The results are shown in Table I. The experiment shows the titer of the anti-

TABLE I.

Titration of the Anti-Human Amboceptor with Guinea Pig Complement.

Amount of serum to each tube.*	Anti-human hemolytic immune serum.	
	No. 633 (rabbit).	No. 634 (rabbit).
cc.		
0.01	Complete hemolysis.	Complete hemolysis.
0.007	" "	" "
0.005	" "	" "
0.004	" "	" "
0.003	" "	" "
0.002	" "	" "
0.0015	" "	" "
0.001	" "	" "
0.0007	" "	" "
0.0005	Partial hemolysis.	" "
0.0004	No "	Partial hemolysis.
0.0003	" "	No "
0.0002	" "	" "
0	" "	" "

* Each tube contained guinea pig complement 0.04 cc. and 1 per cent human corpuscle suspension 1 cc.

human hemolytic immune serum No. 633 to have been 0.0007 cc. and that of No. 634, 0.0005 cc. in the presence of 0.04 cc. of guinea pig complement. 0.1 cc. of the guinea pig serum alone was itself somewhat hemolytic.

From the results recorded in the first part of Table II it appears that in the presence of 0.1 cc. of a fresh human serum at least 0.005

TABLE II.

Titration of the Anti-Human Amboceptor with Human Complement.

Serum 3 + amboceptor.*		Result.
cc.	cc.	
0.1	+ 0.1	Complete hemolysis.
0.1	+ 0.05	" "
0.1	+ 0.03	" "
0.1	+ 0.02	" "
0.1	+ 0.01	" "
0.1	+ 0.005	" "
0.1	+ 0.003	Considerable "
0.1	+ 0.002	No "
0.1	+ 0.001	" "
0.5	+ 0.02	Complete hemolysis.
0.3	+ 0.02	" "
0.2	+ 0.02	" "
0.1	+ 0.02	" "
0.05	+ 0.02	Slight hemolysis.
0.02	+ 0.02	No "

* Human serum as complement. 1 per cent human corpuscle suspension 1 cc. in each tube.

cc. of the anti-human amboceptor No. 633 was needed to produce complete hemolysis; that is, about seven times the amount required in the case of guinea pig complement. In other words, the activity of human complement is nearly one-seventh that of the guinea pig serum.

In the second part of the table it is shown that 0.05 to 0.02 cc. of the same fresh human serum was unable to cause complete hemolysis even in the presence of 0.02 cc. of the amboceptor No. 633, which is an equivalent of thirty minimal lytic doses when calculated on the basis of 0.04 cc. of the guinea pig complement. It is evident that

the amount of amboceptor required to dissolve the human corpuscles in the presence of human complement is many times that necessary with guinea pig complement.

The next point is to determine whether or not average fresh human serum contains enough complement to produce complete hemolysis. Upon this fact depends the possibility of utilizing the human com-

TABLE III.

Quantitative Relation between Human Complement and Anti-Human Amboceptor.

Serum No. (48 hrs. old).*	Human serum 0.1 cc.		Human serum 0.05 cc.	
	Amboceptor 0.005 cc.	Amboceptor 0.01 cc.	Amboceptor 0.005 cc.	Amboceptor 0.01 cc.
1	Considerable hemolysis.	Complete hemolysis.	No hemolysis.	Complete hemolysis.
2	No hemolysis.	No hemolysis.	" "	No hemolysis.
3	Considerable hemolysis.	Complete hemolysis.	" "	Complete hemolysis.
4	" "	" "	" "	" "
5	" "	" "	" "	" "
6	Complete hemolysis.	" "	" "	" "
7	Considerable hemolysis.	" "	" "	" "
8	No hemolysis.	No hemolysis.	" "	No hemolysis.
9	" "	" "	" "	" "
10	Considerable hemolysis.	Complete hemolysis.	" "	Complete hemolysis.
11	Almost complete "	" "	" "	" "
12	Considerable "	" "	" "	" "
13	Complete hemolysis.	" "	" "	" "
14	Considerable hemolysis.	" "	" "	" "
15	" "	" "	" "	" "
16	" "	" "	" "	" "
17	" "	" "	" "	" "
18	" "	" "	" "	" "
19	" "	" "	" "	" "
20	" "	" "	" "	" "

* Each tube contained 1 per cent washed human corpuscle suspension 1 cc.

plement in the serodiagnosis of syphilis. Twenty sera were tested for this purpose. All except three of them (Nos. 2, 8, and 9, Table III) contained sufficient complement in 0.05 to 0.1 cc. to cause complete hemolysis in the presence of 0.01 cc. of the anti-human amboceptor No. 633. No hemolysis occurred, however, in any tube containing 0.005 cc. of the amboceptor and 0.05 cc. of human serum.

There was considerable, and in some cases complete hemolysis in the tubes containing the same amount of the amboceptor but 0.1 cc. of the human serum.

For comparison these twenty sera were also tested upon sheep corpuscles (1 cc. of a 1 per cent suspension) for their natural anti-sheep hemolysin. The results obtained show that 0.05 cc. and 0.1 cc. both dissolved the sheep corpuscles in all except the three specimens (Nos. 2, 8, and 9) which failed to dissolve the human corpuscles in the presence of anti-human amboceptor. This means that there were at least three sera out of twenty which did not contain sufficient complement.

Out of 1,250 specimens of fresh human sera so far examined, 1,157 contained enough complement to produce complete hemolysis of 1 cc. of a 1 per cent suspension of human corpuscles in a dose of 0.1 cc., with the addition of 0.01 cc. of anti-human amboceptor (one unit), within a period of 20 to 30 minutes at 37°C. in a water bath thermostat. 72 specimens caused a partial hemolysis, and 21 no hemolysis.

Additional amboceptor in doses of from one-half to one ordinary minimal hemolytic unit to the partially hemolyzed tubes produced complete hemolysis on a further incubation of 15 minutes or longer. In the case of the sera, however, which showed no complementary action in the first combination, the addition of an extra quantity of the amboceptor caused only a tardy partial hemolysis or no hemolysis. From the serological standpoint we encounter at least three groups of human sera, those which contain the average amount of complement, those which contain a subnormal quantity (hypocomplementosis), and those which contain no complement (acomplementosis). Occasionally one meets with a fourth group in which the activity of the complement is unusually strong (hypercomplementosis). It is highly important to take these facts into account in testing human sera by the Bordet-Gengou reaction or the Wassermann reaction in syphilis.

The next step was devised in order to determine whether the insufficiency of complement could not be supplemented by the addition of an adequate quantity of fresh active serum. That this is easily accomplished was soon proved.

Mode of Utilization of Human Complement for the Serodiagnosis of Syphilis.

The experiments discussed above made it evident that in the majority of fresh human sera there is sufficient complement to cause a complete and prompt hemolysis of human corpuscles in the presence of an adequate quantity of the specific anti-human hemolytic amboceptor. Moreover, in cases where there is not enough complement, an active human serum may be added as a supplement. In fact, we are now in a position to produce complete hemolysis, and from this point it is only another step to test the presence or absence of a complement-fixing principle in a given specimen of human serum. One merely measures out a definite amount of the suspected serum into two tubes and then adds to one of the two an adequate amount of the antigen suspension. Both tubes are incubated for 30 minutes at 37°C. in a water bath; then the human corpuscular suspension and the anti-human hemolytic amboceptor are introduced into both tubes and the contents are well mixed by shaking. The tubes are once more incubated for 30 minutes at 37°C., and then after another 30 minutes or so at room temperature the result is read. It is necessary to shake the tubes two or three times during the incubation. No result should be taken as final unless the control tube without the antigen shows complete hemolysis. If hemolysis is incomplete at the end of the period indicated, adequate modification, which will be described later, must be made.

Procedure for the Examination of Human Sera Not More than 48 Hours Old.

It must be made clear in the beginning that specimens should be examined as soon as practicable, preferably within 24 hours after they are withdrawn from the patients. After 48 hours, even when the specimens are kept in the refrigerator, the complement gradually disappears from the serum. Sera kept for more than 3 days in a refrigerator must be tested by a special technique, to be given later. Specimens tinged deeply with hemoglobin give unsatisfactory results and should be rejected. Table IV indicates the amounts of reagents to be used in the test for fresh human sera and other details regarding it. The entire set of tubes should be duplicated for each specimen.

TABLE IV.
Procedure for Examining Fresh Human Sera.

Tube.	1st step.	2nd step.	3rd step.	4th step.	Final step.
Determinative tube (front row).	Patient's serum (fresh) 0.2 cc. Antigen 0.1 " 0.9 per cent saline so- lution 1 " Patient's serum (fresh) 0.2 " Antigen omit- ted. 0.9 per cent saline so- lution 1.1 "	First incubation at 37°C. for 30 min. in water bath or 1 hr. in air ther- mostat.	Both tubes receive 0.1 cc. of anti-hu- man amboceptor, representing 1 hemolytic unit, and 0.1 cc. of 10 per cent human corpuscular sus- pension. Total volume 1.5 cc. Contents are well mixed by shak- ing.	Second incubation at 37°C., same as first, except that tubes are shaken three times dur- ing the period.	Reading of results after tubes have stood 30 min. at room temperature.
Control tube (back row).					

Positive and Negative Controls.

As in any other serodiagnostic procedure, each serum tested must be accompanied by a positive and a negative serum in order to control the reliability of the reagents. In a well appointed laboratory, where many tests are being made daily or every other day, the necessary positive and negative sera will be furnished by the tests of the previous occasion.

Varieties of Irregular Reactions and Their Adjustment.

Reference has already been made to the possible deficiency of complement in certain specimens of fresh human sera. With these hypo-complementary sera hemolysis in the control tubes without antigen proceeds slowly and remains incomplete at the end of the second incubation. To these sets of tubes, both the determinative and the control, an additional hemolytic amboceptor unit is introduced in order to reinforce the hemolytic activity of the complement. In cases in which hemolysis is marked only one-half the unit is added, but when hemolysis is slight a whole unit of the amboceptor is needed. The tubes should then be put in another rack and subjected to further incubation until complete hemolysis occurs in the control tubes (without the antigen). 15 minutes or longer may be required. Specimens which fail to hemolyze or in which hemolysis is incomplete, even with the additional amboceptor should be tested again by adding to them a quantity of fresh serum which has been shown to contain an average complement and at the same time to be devoid of any syphilitic fixation substance (negative serum). In these cases 0.1 cc. of the complementary serum is used with 0.2 cc. of the acomplementary serum. The mixture is then tested in the same way that any fresh serum is tested.

Procedure for the Examination of Human Serum More than 48 Hours Old.

As previously stated, specimens of human serum which have stood in a refrigerator longer than 48 hours are inconstant in their complementary activity, and many are markedly deficient. As a rule

sera not more than 72 hours old which have been kept constantly in the refrigerator at 4–6°C. still contain enough complement to make the test possible. It is best, however, to inactivate all sera whose complementary activity is no longer a certain factor, supplementing them with active human complement from negative fresh sera. If inactivation is complete (55°C. for 30 minutes), the sera are rendered free of the remnant of their native complement, and what is added later is of known and uniform quantity. 0.1 cc. of the complement serum (previously tested) is added to 0.2 cc. of the inactivated serum and the mixture then tested like any fresh serum. Table V gives the details of the procedure.

Procedure for the Examination of Cerebrospinal Fluids.

This procedure is comparatively easy and gives an entirely satisfactory result. It differs from that used for inactivated sera only in one respect, that of the quantity of the specimen used, which may vary from 0.2 to 0.5 cc. No inactivation is required, as the cerebrospinal fluid contains no complement, and 0.1 cc. of active negative human serum (previously tested) is added as complement (Table VI).

A Method of Preserving the Complement of Fresh Human Serum by Means of Sodium Acetate.

Rhamy³ first observed that guinea pig complement remains active for a long time when mixed with sodium acetate in a strength of approximately 6 per cent of the acetate in the mixture. He recommends mixing 4 parts of the complement with 6 parts of a 0.9 per cent sodium chloride solution containing 10 per cent sodium acetate.

Human complement can also be kept active for some time by adding sodium acetate in a similar proportion. Specimens of human serum which have been mixed with the acetate can be satisfactorily tested after several days without the aid of complement from another source, since the complement remains active for at least 4 days at room temperature (18°C.). Therefore any serum which cannot be examined within 48 hours may be mixed with the acetate saline solution while perfectly fresh. If a good refrigerator is not accessible the specimen

TABLE V.
*Procedure for Examining Inactivated Human Sera.**

Tube.	1st step.	2nd step.	3rd step.	4th step.	Final step.
Determinative tube (front row).	Patient's serum (in- activated) 0.2 cc. Active negative serum 0.1 cc.† Antigen 0.1 " 0.9 per cent saline solution 0.9 cc. Patient's serum (in- activated) 0.2 cc. Active negative serum 0.1 cc.† Antigen omitted. 0.9 per cent sa- line solution 1 cc.	First incubation for 30 min. in water bath or 1 hr. in air thermostat.	Anti-human ambo- ceptor 1 unit in 0.1 cc. of 10 per cent human cor- puscular suspen- sion, 0.1 cc. Total volume 1.5 cc. Contents are well mixed.	Second incubation same as first ex- cept that tubes are shaken three times during in- cubation.	Reading of results within 30 min. af- ter removal of tubes from incu- bator.
Control tube (back row).					

* This applies also to unheated sera which have too little complement from the beginning or have lost complement on standing. Old sera are often anticomplementary and for such specimens only 0.1 cc. is indicated.

† A second complete test of this serum should accompany the test of the serum to which it is added as complement.

TABLE VI.
Procedure for Examining Cerebrospinal Fluid.

Tube.	1st step.	2nd step.	3rd step.	4th step.	Final step.
Determinative tube (front row).	Cerebrospinal fluid 0.2 cc.* Active negative serum 0.1 cc.† Antigen 0.1 " 0.9 per cent saline solution 0.9 cc. Cerebrospinal fluid 0.2 cc.	First incubation same as in Tables IV and V.	Anti-human amboceptor 1 unit in 0.1 cc. of 10 per cent human corpuscular suspension, 0.1 cc. Total volume 1.5 cc. Contents are mixed by shaking.	Second incubation same as first except that contents of tubes are shaken three times during incubation period.	Reading of results within 30 min. after removal of tubes from incubator.
Control tube (back row).	Active negative serum 0.1 cc.† Antigen omitted. 0.9 per cent saline solution 1 cc.				

* Graduated quantities of from 0.2 to 0.5 cc. may be used in certain cases, the amount of saline solution being so adjusted as to make the total volume 1.3 cc.

† A second complete test of this serum should accompany the test of the serum to which it is added as complement.

may always be acetated, since the presence of the acetate does not interfere with the test (Rhamy).

The acetate may be used as preservative for the serum in either of two ways. A sterile solution, containing 0.9 per cent sodium chloride and 10 per cent sodium acetate, may be mixed in equal parts with the serum after separation from the clot; or 0.5 cc. of a sterile concentrated acetate solution (50 per cent) in 0.9 per cent saline solution may be placed in a graduated centrifuge tube and the blood drawn from the patient directly into the tube up to the 5 cc. mark, the whole being then shaken thoroughly. Coagulation takes place as usual, and the clot is just as firm as in a control tube in which 0.5 cc. of saline solution is added to the same amount of blood. There is no disturbing effect from the presence of the acetate in the serum, either when it is fresh, or when it has stood for several days. The serum acetate mixture may be kept in the refrigerator, where it will retain complement activity longer than at room temperature; in the latter, activity is preserved for several days. Once the acetate serum has become inactive by long standing, it can still be tested by adding fresh human complement.

The method of mixing clear serum with the saline acetate solution is much to be preferred to the direct mixing of the blood and concentrated acetate solution.

Preparation of Reagents.

Although the details with regard to the reagents used in conducting the present test are essentially the same as those described in earlier publications^{6, 16} dealing with the anti-human heterohemolytic system (the use of guinea pig complement with anti-human amboceptor and human corpuscles), it nevertheless seems desirable to summarize them briefly here. Antigen, anti-human hemolytic amboceptor, and a suspension of human blood corpuscles are the reagents required to test the patient's serum.

Antigen.—The preparation, titration for its antigenic properties, and preservation of the antigen are described in other publications dealing with the subject in minute detail.⁶ Suffice it to say that the acetone-insoluble fraction of tissue

¹⁶Noguchi and Bronfenbrenner, Biochemical studies on so-called syphilis antigen, *J. Exp. Med.*, 1911, xiii, 43.

lipoids is recommended, 0.1 cc. of a 1:10 emulsion with 3 per cent methyl alcohol solution (stock) and 0.9 per cent saline solution being used for each test. No preparation which hemolyzes in 0.4 cc. or interferes with complement in the same dose should be used. The antigen should be effective in doses of 0.02 cc. of the emulsion. In the actual test at least five antigenic units are employed in order that no positive reaction may be overlooked. The methyl alcohol stock solution remains active indefinitely at room temperature, and a saline emulsion of 1:10 strength may be made up at any time. The latter, when kept in a refrigerator, remains unchanged for several days, although it is best to make a fresh emulsion on the day that tests are to be made.

Amboceptor.—The production of the anti-human amboceptor is one of the most important parts of the present method. Rabbits, when immunized with thoroughly washed human corpuscles in sufficiently large quantities, yield a powerful serum, which is able to produce complete hemolysis of 1 cc. of a 1 per cent suspension of human corpuscles in doses of from 0.01 to 0.005 cc. This point will be discussed at greater length later. The usual amount of this reagent used in the test is 0.1 cc., which is so made as to represent one hemolytic unit in the presence of 0.1 cc. of fresh human serum and against 1 cc. of the 1 per cent corpuscular suspension, hemolysis becoming complete within 20 to 30 minutes at 37°C. in a water bath or 1 hour in an air thermostat.

Corpuscular Suspension.—0.1 cc. of a 10 per cent suspension is best suited for the purposes of the test. The suspension is prepared as follows: 1 part of the washed corpuscles from any individual (it is convenient to use corpuscles obtained from one of the patients being bled for test serum) is mixed with 9 parts of 0.9 per cent saline solution. A few cubic centimeters of blood are drawn into a centrifuge tube containing an equal volume of sodium citrate solution (2 per cent in 0.9 per cent saline solution) and the mixture is repeatedly centrifuged with changes of saline solution until there is no more serum in the supernatant fluid. The final sediment of corpuscles is suspended in a quantity of saline solution equal to the original quantity of the blood. The corpuscular suspension thus prepared should be used when fresh and when not in use should be kept in a refrigerator, where it can be preserved for a period of about 72 hours. A suspension older than 72 hours should not be used, however, because the use of corpuscles with diminished resistance to hemolysis may cause the masking of a possible weak positive reaction.

Utilization of the Patient's Corpuscles.—For any well equipped laboratory the preparation of a corpuscular suspension of a definite concentration by the above method offers no difficulty. There may be occasions, however, for example on board ship or in field hospitals, when no centrifuge is available. Under these conditions the preparation of a uniform corpuscular suspension for an entire set of tests is not possible, and to meet this sort of emergency the utilization of the patient's own corpuscles for testing his serum is recommended. As has already been emphasized, the serum of the patient to be examined must be perfectly fresh and the corpuscles which can be liberated by gently stirring the clot are correspondingly fresh and can be used as the indicator of hemolysis. The fol-

lowing technique gives the best result: 0.4 cc. of the fresh clear serum from the tube containing the coagulated blood is measured out and put into one of the two tubes used for the test. 2 cc. of 0.9 per cent saline solution are added, making a total volume of 2.4 cc. After putting the remaining clear serum into a tube for future use, the clot is gently shaken, by means of a medium sized pipette, to liberate enough corpuscles to tinge the serum dilution to the desired color value (suspension). The standard of suspension aimed at is 1 per cent of corpuscles, and after a certain amount of practice no difficulty is experienced in detecting the difference between a 1 per cent and a 1.5 per cent suspension by this means. A 1 per cent suspension is an opaque fluid with a yellowish red hue. As the concentration of the suspension increases, the red becomes more predominant over the yellow tint. Having made a mixture of 0.4 cc. of the fresh serum and approximately a 1 per cent corpuscular suspension in a total of 2.4 cc., one divides this quantity into two equal portions by measuring out 1.2 cc. into the second tube of the set. One of the two tubes receives the antigen 0.1 cc., and the other receives the saline solution 0.1 cc., and serves as the control without the antigen. Both are incubated for 30 minutes in a water bath or 1 hour in an air incubator. 0.1 cc. of the anti-human amboceptor, representing one hemolytic unit, is then added to both tubes, and after thorough mixing the tubes are again incubated, as in all other procedures.

When the test is done in this way, the corpuscles are, of course, present from the beginning, instead of being added after the first incubation simultaneously with the amboceptor, as in procedures in which corpuscles from other patients are used. But in any procedure the corpuscles may be introduced from the beginning and the final result remain the same.

Tschernogubow⁸ once proposed the use of a suspension of the patient's blood directly diluted in saline solution in a ratio of 1 drop to 1 cc. of saline. This was to serve as the source of complement, corpuscles, and, if present, the fixing substance. By careful scrutiny, however, one soon discovers the enormous disproportion among the various elements concerned. For example, the amount of serum probably present in 1 drop of blood, which would perhaps be 0.07 cc., would be approximately 0.035 cc. at most (about one-sixth the amount used in the writer's system), while the corpuscular suspension approaches 7 per cent (seven times the concentration in the writer's system). The amount of syphilitic antibodies present would be too small to make possible the detection of a weak positive reaction, and the minuteness of complement present in such a mixture precludes any practical possibility. Even an enormous amount of the amboceptor fails to complete hemolysis of such a concentrated

suspension of the corpuscles. Moreover, the mixture forms a loose gelatinous fibrin, involving the whole contents, making it impossible to stir by shaking. When the first fibrin is removed, a second may form on further standing. Such a method cannot be used, and Tschernogubow himself soon abandoned it.

Emery⁹ employs active serum with the cholesterolized alcoholic extract of heart muscle, disregarding the possibility of obtaining a false positive fixation with certain non-syphilitic sera. The amount of the patient's serum is minute, but the concentration of the corpuscles (20 per cent of the firmly packed sediment after centrifugation) is almost six times that used in the method being proposed. There is no economic gain in Emery's method, therefore, since the amount of amboceptor required is no less than that used in the proposed method. Moreover, the manipulation of minute quantities of various factors by Wright's capillary technique requires a considerable degree of skill as compared with the ease with which regular graduated pipettes can be handled.

Butler and Landon¹⁰ and Myer¹¹ inactivate the patient's serum before the test and add fresh negative human serum from a non-syphilitic individual as complement. They recommend the use of sensitized human corpuscles and the acetone-insoluble tissue lipoids (Noguchi). Their method is decidedly more rational than Emery's and seems to have given satisfactory results in 300 cases so far reported.¹⁶ They also employ Wright's capillary technique.

Thompson¹² uses fresh human serum, with preliminary titration of each specimen for its complement activity. This procedure seems to be unessential, since the majority of specimens already contain enough complement. The amount of corpuscles used is 0.2 cc. of a 2 per cent suspension. A stronger concentration would give a more distinct reaction.

Results of Practical Application of the Test.

The writer has been able, up to the present time, to examine 1,331 specimens of blood and 52 cerebrospinal fluids.¹⁷ Of 1,118 specimens of sera from these sources 517 gave a positive and 601 a negative

¹⁷I wish to express my appreciation to the members of the staffs of several hospitals through whose cooperation this work was made possible.

reaction, the results conforming to those reported by the serological departments of the various hospitals. Of 132 specimens from psychiatric cases, 54 were from general paralysis cases, and all except 2 gave a strongly positive reaction. Among other psychoses, including 75 cases of dementia præcox, 3 of alcoholic psychosis, 3 of imbecility, 3 of senile psychosis, 6 of arteriosclerosis, 1 of manic-depressive insanity, and 1 paranoic condition, there were only 2 positive reactions, these occurring among the dementia præcox cases. The reactions with 81 inactivated sera agreed with those obtained by others with the same material. 20 cerebrospinal fluids from cases of general paralysis gave a strongly positive reaction, while 32 specimens from other non-syphilitic cases showed a negative reaction. The statement will perhaps bear repeating that of 1,250 fresh human sera complement was deficient in 93 specimens, which had to be examined either by means of additional amboceptor or by supplying active human complement from fresh negative sera. This special adjustment with fresh sera is one which demands particular attention on the part of serologists adopting the method.

Quantitative Consideration of the Complement Fixation Test.

In an ideal method for the serum diagnosis of syphilis every ingredient should be separately controllable by accurate titration. This is possible with the anti-human heterohemolytic system (Noguchi), in which the amount of guinea pig complement is accurately measured and added to a definite quantity of serum, whose native complement plays no part in the reaction, or has been removed by inactivation, and in which the antigen, anti-human amboceptor, and corpuscles are equally definite, no factor being present which can give rise to a quantitative disturbance. The homohemolytic system, upon critical examination, will be seen also to be capable of equal accuracy. The possible sources of error and the methods of adjusting them are discussed below.

The Frequency and Extent to Which a Positive Reaction May Be Masked by an Excess of Complement.—Only 2 per cent of several hundred specimens examined in this study were found to contain an extra unit of complement activity. None contained three units.

An excess of complementary activity may cause a positive serum to give a weakly positive or even a negative reaction, but only when the so called antibody content of the serum is less than one fixing unit. For example, a specimen containing half of an antibody unit in the presence of two complement units may give a negative result. No change in the reaction can occur, however, from an excess of less than two complement units in the presence of $1\frac{1}{2}$ units of syphilitic antibody. Error from this source is therefore extremely rare, and the reaction cannot be completely negative when the specimen contains more than one antibody unit.

TABLE VII.

Relation between Various Quantities of Human Complement and of Antibody.

Syphilitic serum (active).		Fresh negative human serum as complement.						
Amount.	No. of units of antibody.	0.1 cc.	0.125 cc.	0.15 cc.	0.2 cc.	0.25 cc.	0.3 cc.	0.4 cc.
cc.								
0.008	0.5	+++	+++	++	++	+	+	—
0.016	1.0	++++	++++	++++	++++	+++	+++	+
0.024	1.5	++++	++++	++++	++++	++++	+++	++
0.032	2.0	++++	++++	++++	++++	++++	++++	+++
0.04	2.5	++++	++++	++++	++++	++++	++++	++++
0.048	3.0	++++	++++	++++	++++	++++	++++	++++
0.064	4.0	++++	++++	++++	++++	++++	++++	++++

Table VII records experiments in which the relation between various quantities of human complement and of antibody was determined. The amount of anti-human amboceptor used was that which produced complete hemolysis of 1 cc. of a 1 per cent suspension of human corpuscles in the presence of 0.1 cc. of fresh human serum (one complement unit) within 20 to 30 minutes at 37°C. (water bath). Table VII shows that the addition of quantities of the human complement ranging from 0.1 to 0.2 cc. did not materially change the + + + + reaction when combined with 0.016 cc. of syphilitic serum. The reactions became somewhat weaker, however, when 0.15 and 0.2 cc. of the complement were used with 0.008 cc. of the serum. When the amount of the syphilitic serum was increased to 0.024 to 0.032 cc. or more, the reaction was + + + + against 0.3 cc. of the complement, and

0.04 cc. of serum prevented hemolysis in the presence of 0.4 cc. of complement. The reduction in the degree of positive reaction caused by the variations of 0.1 to 0.2 cc. of the human complement is insignificant and has no serious effect upon the ultimate result when the serum contains more than $1\frac{1}{2}$ antibody units. The apparent disproportion of complement in the routine amount of 0.2 cc. of the fresh serum is well balanced by the antibody content, which is four to five times that of the same serum inactivated. For that reason only 0.1 cc. of human serum is added to 0.2 cc. of inactivated serum. This proportion is similar to that of the guinea pig complement and inactivated serum used in the Wassermann system and in the anti-human heterocomplement system (Noguchi). But the addition of 0.2 cc. of the human complement does not mask a strongly positive reaction (++++).

The Possibility of Interference by Negative Serum in Complement Fixation in the Homohemolytic System.—The use in the homohemolytic system of fresh negative serum as complement when there is no complement or too little in the specimen to be examined, while entirely analogous to the use of fresh guinea pig serum as complement for an inactivated patient's serum, may rouse apprehension as to the possibility of reduction in the degree of fixation by this comparatively large amount of human complement, owing to its indifferent serum constituents. The experiments recorded in Table VIII, in which human and guinea pig complement were studied in parallel series, show that the addition of an inactivated negative serum to a syphilitic serum does not cause any so called complementoid blocking of the complement fixation of any significance, and that no error can result from this source.

All the other experiments of this sort with syphilitic sera gave similar results. It was found, however, that a syphilitic serum containing less than one-half an antibody unit caused less inhibition in the tubes to which more than 0.3 cc. of the inactivated negative human serum had been added, but never completely masked the reaction. There was no appreciable difference in the tubes containing 0.1 or 0.2 cc. of the inactivated serum and that containing none. The addition, however, of inactivated guinea pig serum (56°C.) to a syphilitic serum produces marked weakening of the fixation reaction, and even com-

TABLE VIII.

Effect of Inactivated Negative Human Serum upon the Complement Fixation.

	With human complement.	Results.	With guinea pig complement.	Results.
With inactivated syphilitic serum.	Syphilitic serum (56°C.) containing 1 antibody unit 0.2 cc. } Human complement (active serum) 0.1 " }	All gave complete fixation, regardless of the addition of the inactivated negative serum.	Syphilitic serum (56°C.) containing 1 antibody unit 0.2 cc. } Guinea pig complement 40 per cent 0.1 " }	All gave complete fixation, no interference being observed from the addition of the inactivated negative human serum.
	The same + negative human serum (56°C.) 0.1 "		The same + negative human serum (56°C.) 0.1 "	
	The same + negative human serum (56°C.) 0.2 "		The same + negative human serum (56°C.) 0.2 "	
	The same + negative human serum (56°C.) 0.3 "		The same + negative human serum (56°C.) 0.3 "	
	The same + negative human serum (56°C.) 0.4 "		The same + negative human serum (56°C.) 0.4 "	
With active syphilitic serum.	Active syphilitic serum, containing 4 antibody units 0.2 cc.	Complete fixation in all.		
	The same + negative human serum (56°C) 0.1 "			

TABLE VIII—*Concluded.*

	With human complement.	Results.	With guinea pig complement.	Results.
With active syphilitic serum.	The same + negative human serum (56°C.) 0.2 cc.	Complete fixation in all.		
	The same + negative human serum (56°C.) 0.3 "			
	The same + negative human serum (56°C.) 0.4 "			

plete blocking when more than 0.3 cc. of that serum is added to one syphilitic antibody unit. This confirms earlier observations.¹⁸

Result of the Presence of an Excess of Amboceptor in the Complement Fixation Test.—The disturbance resulting from an excess of amboceptor in any complement fixation test has been repeatedly pointed out and is recognized by impartial workers as inherent in the anti-sheep hemolytic system. Removal by absorption of the natural anti-sheep amboceptor from each specimen of serum prior to test has been advocated, but for obvious reasons is impracticable when several dozen specimens must be examined at one time.

In Table IX are recorded the results obtained in parallel series of tests,¹⁹ the anti-sheep system with guinea pig complement being used in one series, and the anti-human homocomplement system in the other. These experiments confirm earlier observations²⁰ that a

¹⁸ Noguchi and Bronfenbrenner, The interference of inactive serum and egg-white in the phenomenon of complement fixation, *J. Exp. Med.*, 1911, xiii, 92.

¹⁹ These tests were carried out by Major Felix R. Hill, Captain George L. Schadt, and Lieutenant Ralph R. Simmons in this laboratory.

²⁰ Noguchi, Die quantitative Seite der Serodiagnostik der Syphilis, *Z. Immunitätsforsch., Orig.*, 1911, ix, 715.

positive serum containing 1 antibody unit can become completely negative when 4 amboceptor units are used, or 3 antibody units with 20 amboceptor units. Table IX shows that 1 antibody unit is made negative by 6 amboceptor units and 3 antibody units by 10 amboceptor units with the Wassermann system, while with the anti-human homocomplement system nearly twice the amount of amboceptor is required to produce the same effect.

The phenomenon just described assumes practical importance in any system in which are used foreign blood corpuscles for which human serum normally contains varying amounts of natural hemolytic amboceptor, in which case there is a possibility of an excess of amboceptor in the test. It does not occur in an anti-human hemolytic

TABLE IX.
Reversion of Reaction through Excess of Amboceptor.

No. of units of amboceptor.	Syphilitic antibody 1 unit.		Syphilitic antibody 3 units.		Syphilitic antibody 10 units.	
	Wassermann anti-sheep system.	Anti-human homocomplement system.	Wassermann anti-sheep system.	Anti-human homocomplement system.	Wassermann anti-sheep system.	Anti-human homocomplement system.
1	++++	++++	++++	++++	++++	++++
2	++++	++++	++++	++++	++++	++++
3	++	++++	+++	++++	++++	++++
6	—	+++	+	++++	++++	++++
10	—	—	—	++	++++	++++
20	—	—	—	—	+++	+++
40	—	—	—	—	+	+

TABLE X.
Titration of Natural Anti-Sheep Amboceptor in Human Serum.

Sera.	No. of specimens examined.	No. of units of anti-sheep amboceptor in 0.2 cc. of human serum (56°C.), titrated with guinea pig complement.											
		None.	< 1	1	2	3	4	5	6	7	8	>10	
Syphilitic sera	190	19	21	42	21	25	26	17	8	4	3	4	
Non-syphilitic sera.....	111	3	15	25	31	10	12	8	5	1		1	
Normal sera	25	1	3	5	4	3	1	2	2	3	1		
Total.....	326	23	39	72	56	38	39	27	15	8	4	5	

system, whether guinea pig complement or human complement is used, and especially when the patient's own corpuscles are employed for each specimen. Data which have been collected concerning the amount of natural anti-sheep amboceptor in 326 specimens of human serum are given in Table X.

Tables IX and X explain the possibility of a reduced or reversed reaction with certain syphilitic sera in the anti-sheep system. The frequency and extent of error from this source are much greater than those due to slight variations in complement content, which are readily amenable to quantitative adjustment.

Quantitative Estimation of the So Called Syphilitic Antibody.—If necessary, any strongly positive serum can be titrated by the homo-complement system, the procedure being the same as that recommended for the anti-human heterocomplement system; namely, that of keeping all the other ingredients constant and determining the smallest quantity of the serum which will give complete fixation. For this purpose the serum may be inactivated before titration. To a number of tubes containing 0.1 cc. of the fresh human complement (previously titrated) and the standard antigen (usually contained in 0.1 cc. of a suitable dilution) are added varying amounts (ranging from 0.01 to 0.2 cc.) of the positive human serum (inactivated) to be titrated, and the mixture is made up to 1.3 cc. in each tube. After an incubation at 37°C. for 30 minutes in the water bath or 1 hour in an air incubator, the corpuscle suspension (0.1 cc.) and one unit of amboceptor (0.1 cc.) are added, and a second incubation follows. The smallest quantity of serum which produces complete fixation is taken as one antibody unit.

SUMMARY.

The elimination of the foreign complement and corpuscles from the test for the serodiagnosis of syphilis has been attempted, and the results so far obtained are very satisfactory. Instead of using guinea pig complement, fresh human serum was utilized for the source of complement for the production of hemolysis upon the human corpuscles in the presence of an adequate amount of the specific anti-human amboceptor (prepared in rabbits). Usually 0.1 cc. of fresh

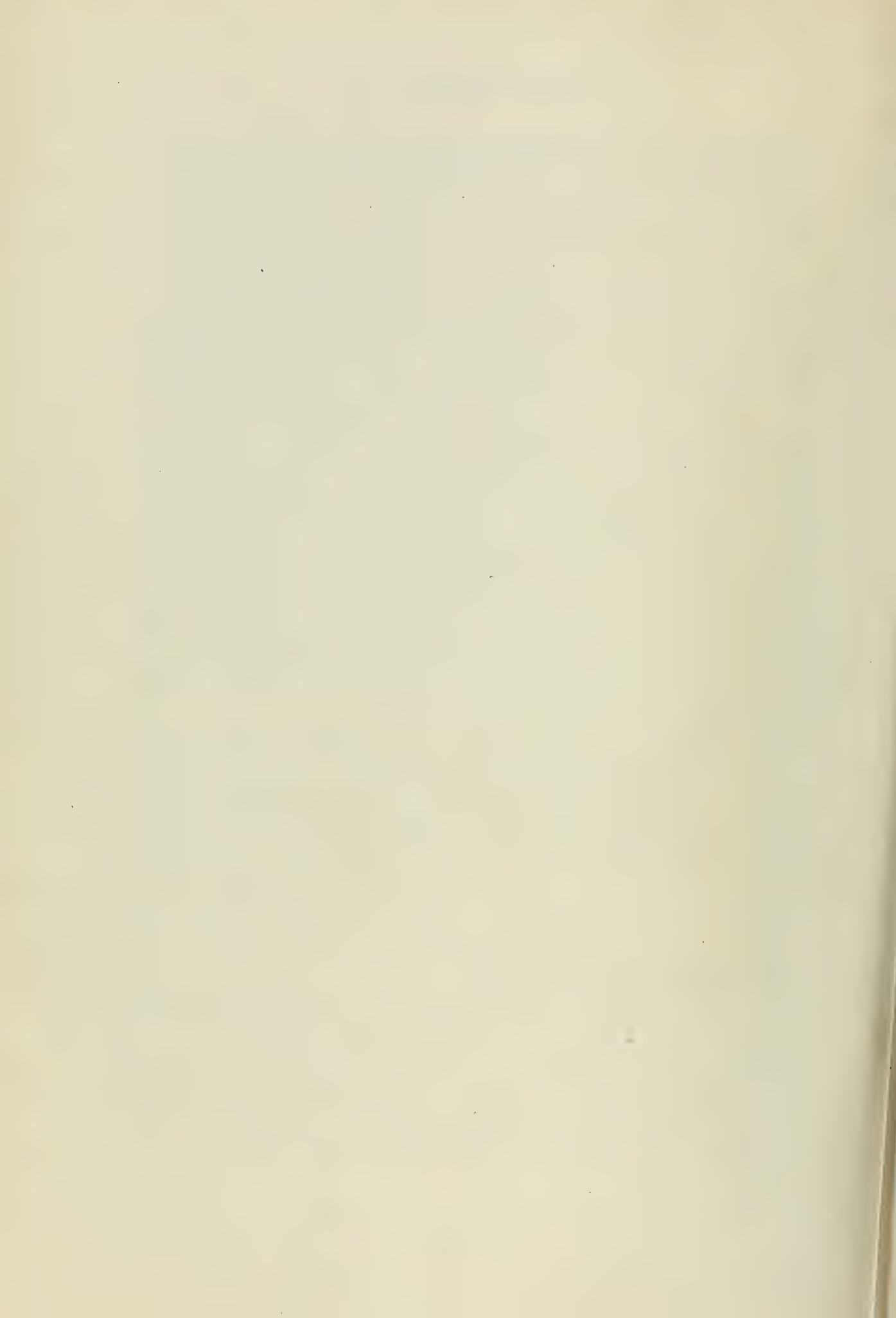
human serum contains enough complement to hemolyze 1 cc. of a 1 per cent suspension of human corpuscles, but the amount of anti-human amboceptor required in this combination is about five to seven times that necessary when guinea pig complement (0.04 cc.) is used. It has also been shown that when a given human serum contains insufficient complement, an adequate quantity (0.1 cc. is usually enough) of another fresh negative serum may be added to supplement it. However, one rarely encounters this group of sera. Inactivated human sera can also be examined by utilizing human complement from another source (the serum must be fresh, active, and negative).

The only drawback to the present method is the comparatively large amount of anti-human hemolytic amboceptor required. It is estimated that 30 to 40 cc. of the anti-human hemolytic immune serum, from one rabbit, of high potency—say 0.005 cc.—would be enough to examine about 3,000 to 4,000 cases (0.01 cc. for each case), whereas if guinea pig complement were used the same amount would cover about 15,000 tests (0.002 cc. for each case). In a large hospital or in the Army there should be no difficulty in preparing any amount of the anti-human hemolytic amboceptor. For example, material for 100,000 tests could be prepared within 1 month in less than 100 rabbits. The amboceptor serum can be used in the fluid state, or, if the titer is high, impregnated into filter papers.

Special attention should be called to the fact that to obtain a powerful anti-human hemolytic amboceptor five to six intraperitoneal injections of corpuscles, thoroughly washed (until there is no trace of serum in the supernatant fluid), in doses of 5, 7, 10, 10, 10, and possibly another 10 cc. of the concentrated suspension (restored to the original volume of the blood) are required.²¹ The bleeding may be done by the 9th or 10th day. The animals may be kept after bleeding for the production of more amboceptor by subsequent injections of the washed corpuscles.

Finally, it should be emphasized that only the acetone-insoluble fraction of tissue lipoids of required standards (Noguchi) should be used when utilizing the human complement in the fixation test.

²¹ Intravenous injections of 3, 3, 3, 4, and 4 cc. of the corpuscular suspension every 4 or 5 days also give excellent results.



AN EXPERIMENTAL STUDY OF VACCINATION AGAINST BACILLI DYSENTERIÆ.

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The occurrence of bacillary dysentery in the armies of Europe, the finding of great numbers of cases of this infection in the tropics, where the amebic form has been regarded as the prevailing type, and the presence of the disease endemically in the United States have stimulated this series of investigations.

Attempts have been made at immunization against bacillary dysentery by means of cultures of the dysentery bacilli from the time of their discovery by Shiga up to the present, yet no definite method has been found which made vaccination or active immunization practicable.

An analysis of previous efforts reveals the peculiarities of the *B. dysenteriae* group of organisms. Shiga,¹ in 1898, injected himself subcutaneously with one-twelfth of a killed agar slant culture of *B. dysenteriae* (Shiga). The local reaction was of such intensity that eventually incision was made to evacuate the purulent exudate and to help the absorption of the board-like edema. However, after 10 days agglutinins were demonstrable in his blood. So also Kruse,² who injected himself with 1 cc. of a killed bouillon culture, developed a severe local reaction persisting for a week. After 8 days the agglutinin titer of his serum was 1:800. Rosenthal,³ using a similar antigen upon himself, also developed a marked local and general reaction, but no agglutinins.

These personal experiments were supplemented by animal experiments by others, and the conclusions reached were that simple suspensions of the killed bacilli in saline solution, or killed bouillon cultures, while too toxic for practical use, do nevertheless give rise on inoculation to antibody production.

¹ Shiga, K., *Centr. Bakteriöl., 1te Abt.*, 1898, xxiv, 817, 870, 913.

² Kruse, W., *Deutsch. med. Woch.*, 1900, xxvi, 637; 1901, xxvii, 386; 1903, xxix, 49.

³ Rosenthal, *Centr. Bakteriöl., 1te Abt., Ref.*, 1905, xxxvi, 23.

Attention was next concentrated upon the addition of immune serum to the bacteria, either to sensitize the antigen or to neutralize its toxicity. Shiga,⁴ proceeding on the latter basis, injected killed cultures and immune serum simultaneously for the first injection, following it in 3 to 4 days by a larger dose of the bacilli without serum. He vaccinated 10,000 Japanese in this way and determined that a definite protection was afforded, since the mortality was reduced. The general results, however, were unsatisfactory, as the immunity endured but 3 to 4 weeks. Subsequently Shiga varied the dosage by giving antiserum with the second dose, but although the local and systemic reactions were mitigated, the procedure failed to produce a lasting immunity.

Vaillard and Dopter⁵ and Dopter⁶ employed the Besredka method of sensitizing vaccines and obtained what they regarded as encouraging results, but Lüdke⁷ was unable to detect any advantage in the sensitized vaccine. Lüdke's conclusions are that sensitization not only removes the toxicity of the dysentery bacillus but also destroys its antigenic function. Lucas and Amoss⁸ employed a vaccine consisting of Flexner organisms in combination with immune serum, but on account of the few instances studied no definite conclusions can be drawn. Immune serum alone has been used by Shiga, Dopter, and others as a prophylactic measure, but it is generally conceded that the passive immunity endures usually only about 10 days. Recently Gibson⁹ removed by absorption the so called antibacterial principles of immune serum, leaving the supposed anti-endotoxic elements. The absorbed serum was injected with the killed cultures, and Gibson states that the severe local reaction is abolished and an immunity conferred. It will be shown later that Gibson's serum possesses no advantage over unmodified immune serum.

While the foregoing observations were being made another series of tests revealed that immunization is possible after injection of dysentery bacilli in either changed or unchanged form. Gay¹⁰ ascertained that repeated injections of the organisms in guinea pigs produces an active immunity. Lüdke¹¹ showed that rabbits, after repeated intravenous injections of cultures, develop immune serum of high titer, possessing strong bactericidal powers, agglutinins, complement-fixing antibodies, and precipitins. A similar result follows subcutaneous injection. It is important to note that the agglutinin titer corresponded to the protective value of a given serum. Immunity, though not of such a degree, could also be

⁴ Shiga, K., *Deutsch. med. Woch.*, 1903, xxix, 327.

⁵ Vaillard, L., and Dopter, C., *Ann. Inst. Pasteur*, 1903, xvii, 463.

⁶ Dopter, C., *Ann. Inst. Pasteur*, 1909, xxiii, 677.

⁷ Lüdke, H., *Die Bazillenruhr*, Jena, 1911, 128.

⁸ Lucas, W. P., and Amoss, H. L., *J. Exp. Med.*, 1911, xiii, 486.

⁹ Gibson, H. G., *J. Roy. Army Med. Corps*, 1917, xxviii, 615.

¹⁰ Gay, F. P., *Univ. Penn. Med. Bull.*, 1902-03, xv, 307.

¹¹ Lüdke, H., *Centr. Bakteriöl., 1te Abt., Orig.*, 1905, xxxix, 512, 649.

secured by using autolyzed products of the dysentery bacilli (Neisser and Shiga¹²). The dried powder of dysentery bacilli, suspended in saline solution and injected intravenously into rabbits, has also been used. It produces agglutinins, though not to a great degree. The bacilli digested with pepsin or trypsin have also been tested and an actively bactericidal immune serum has been obtained with them.

All these methods demonstrate the possibility of developing immunity through the injection of the proteins, in some form, of the dysentery bacilli. An analysis of the results will show the advantage of using as antigen bacilli as nearly in their natural state as possible, and such a method conforms with the general principles of active immunization as laid down by Theobald Smith.¹³

With the results of the earlier work before us, keeping in mind the high toxicity of the dysentery bacilli for human beings and considering the importance of employing them as little changed by artificial manipulation as possible, we undertook the following experiments. The objects of the experiments were the finding of a suitable medium for suspending the bacilli, the diminution of their toxic effects, and the retention of their immunizing values.

Several kinds of vaccine of the dysentery bacilli were prepared. It is generally conceded now that the dysentery bacilli may be divided into two main groups, the Shiga and the Flexner. Organisms of either group may produce similar intestinal lesions and clinical symptoms. They are, however, distinguishable by their power to ferment certain carbohydrates and alcohols, and by the fact that the Shiga bacillus yields, together with an endotoxin, what appears to be an exotoxin, while the Flexner bacillus does not. The Shiga group is homogeneous, the Flexner group heterogeneous. In the latter is included the representative type, isolated by Flexner¹⁴ in the Philippine Islands in 1900, the Hiss Y,¹⁵ and the Strong¹⁶ types. The organisms of the Flexner group possess common antibodies: cross-agglutination, as well as cross-protection, is definite. The organisms of the Shiga group are, however, distinguished serologically from those of the Flexner: cross-agglutination and cross-protection do not occur, unless exceptionally, in a small degree.

¹² Neisser, M., and Shiga, K., *Deutsch. med. Woch.*, 1903, xxix, 61.

¹³ Smith, Theobald, *J. Am. Med. Assn.*, 1913, ix, 1591.

¹⁴ Flexner, S., *Philadelphia Med. J.*, 1900, vi, 414.

¹⁵ Hiss, P. H., Jr., and Russell, F. F., *Med. News*, 1903, lxxxii, 289.

¹⁶ Strong, R. P., and Musgrave, W. E., Report of the etiology of the dysenteries of Manila, *Rep. Surg.-Gen. Army*, Washington, 1900, 251.

Hence, in the following experiments the vaccines were made polyvalent, in order to include the antigenic factors of the two groups of organisms. As a representative of the Shiga group a known culture was selected, of definite toxicity. The minimum lethal dose of its toxin fluctuated between 0.05 and 0.1 cc. for a 1,500 to 1,800 gm. rabbit. As a representative of the Flexner group the original Flexner bacillus (the Flexner-Harris, or Philippine strain) was chosen, as well as the Hiss Y, the two organisms present in epidemic and sporadic cases of dysentery. The three organisms were present in the vaccines in equal proportions with reference to their grouping so that an injection would include one-half the total of Shiga organisms, one-quarter of Flexner bacilli, and one-quarter of Y bacilli.

Saline Dysentery Bacilli Vaccine.

The usual saline suspensions of the bacilli were first studied in order to control the antigenic properties of the organism and to confirm previous observations. To this end the following experiment was performed.

Experiment A.—There were employed suspensions of Shiga and Flexner bacilli in isotonic saline solution, of which each cubic centimeter contained 4 billion organisms, killed by heating at 60°C. for 30 minutes, preserved by means of 0.35 per cent tricresol.

Three rabbits, Nos. 1, 2, and 3, were injected subcutaneously with this vaccine. The first dose was 0.25 cc., or 1 billion bacteria, the second dose was 0.5 cc., or 2 billion, and the third was 0.75 cc., or 3 billion. The injections were given at 5 day intervals, under the shaven skin of the abdomen. After 24 hours the three rabbits developed a severe edematous swelling which, as a rule, extended over an area 6 cm. in diameter. There were tenderness and redness, and eventually firm nodules formed. Absorption was slow; usually a week elapsed before the nodule receded to a small, firm, papular mass. Rabbits 1 and 2 succumbed to the third injection.

Agglutination tests were made from time to time, the macroscopic method being employed, and the tubes being kept at 55°C. for 4 to 5 hours.¹⁷ Rabbit 3 showed

¹⁷ It was determined that this method gave much more definite reactions than the old method of incubating for 2 hours at 37°C. and then keeping over night in the ice box. The specificity of the agglutination was not affected by the heat. It was subsequently found that these tests could be left at 55°C. over night with good results.

no agglutination 5 days after the first injection. 5 days after the second injection the serum agglutinated Flexner bacilli in 1:100, but not Shiga bacilli. 7 days after the third injection Flexner and Shiga bacilli were agglutinated in 1:100, and 1 month after the injection the Flexner agglutination was 1:100 and the Shiga negative. Rabbit 2 showed Flexner agglutination 1:100 5 days after the first injection and 1:800 a similar period after the second. This rabbit died 24 hours after the third injection. Rabbit 1 also showed agglutination of 1:100 for Flexner bacilli and 1:50 for Shiga 5 days after the first injection, and 1:200 for Flexner and 1:100 for Shiga 7 days after the third injection. At this time the rabbit died.

The surviving rabbit was injected 1 month after the last vaccination with ten lethal doses of a Shiga culture intravenously. Two controls, similarly injected, died in 18 and 24 hours respectively. The vaccinated animal survived.

From this experiment we may conclude that saline vaccines are too toxic for use: two of the three rabbits succumbed to the injections. At the same time they are capable of developing considerable agglutinins—the agglutination titer was high in all three rabbits. They also afford protection to many times the fatal dose of the Shiga bacillus. With respect to the toxicity, the antigenic property, and the protective effect, the previous observations have been confirmed.

Vaccines Suspended in Chemical Solutions Other than Saline.

A chemical solution was first sought which would approach as closely as possible the concentration and acidity of tissue fluids. For this purpose Henderson's phosphate solution¹⁸ was employed. It contains a 1 per cent concentration of monosodium phosphate 1 part, and disodium phosphate 9 parts.

Experiment B.—The bacilli in the vaccine were prepared in the usual manner and added to the phosphate solution. Three rabbits (Rabbits 4, 5, and 6) were injected in the same manner and with the same dosage as in Experiment A. In none of them was there any local reaction. Rabbits 4 and 5 died after developing paralysis of both anterior and posterior extremities. In none of this series were agglutinins demonstrable. Rabbit 6 alone survived the three injections and was injected 1 month after the last injection with ten lethal doses of a Shiga culture intravenously. The animal succumbed, together with the controls.

¹⁸ Henderson, L. J., *J. Biol. Chem.*, 1909–10, vii, 29.

This method was unsuccessful, since neither agglutinins nor protection developed, and although the local toxicity was diminished, the systemic toxicity was apparent.

The next trial was based on the possibility that the irritation following an injection of the vaccine might be due to acid production in the tissues. Hence a new vaccine was prepared, an alkaline medium being used, consisting of lime-water (aqueous solution of calcium hydroxide), for suspending the killed bacilli.

Experiment C.—Three rabbits (Nos. 7, 8, and 9) were injected in the usual manner (see Experiment A). Rabbit 8 died after the first injection, of typical dysentery intoxication. No local reaction was seen in any of this series. No agglutinins were produced. The two surviving rabbits showed no protection against fatal doses of Shiga and Flexner type cultures.

With alkaline suspensions, then, no agglutinins were produced, nor was protection afforded. Although there was no local reaction, the systemic toxicity was not diminished.

From these experiments we may conclude that the presence of certain chemicals in a vaccine may eliminate its local irritating effect but at the same time will remove its antigenic power and simultaneously increase its systemic toxic effects.

Immune Serum Alone and with Vaccines.

As a control of the experiments to follow, one series of rabbits was injected with polyvalent antidysenteric serum alone.

Experiment D.—Rabbit 10 was injected with 1 cc. of polyvalent antidysenteric serum subcutaneously three times at 5 day intervals. There was no local reaction. No agglutinins were demonstrable. 1 month after the last injection there was no protection against ten lethal doses of a Shiga culture. Repetition of this experiment afforded similar results.

The immune serum alone is incapable of producing agglutinins in dilutions of 1:25 to 1:50, nor is there protection at the end of a month. Hence, whatever results in agglutinin production, protection, or local reaction occurred in the following series are to be ascribed to the bacterial vaccine and not to the immune serum. The uncertainty of duration of the effect of the prophylactic injection of immune serum was incidentally demonstrated.

The next experiment was undertaken to show the effect of the ordinary saline suspension vaccines combined with the use of polyvalent antidysenteric serum.

Experiment E.—The dosage of the vaccine and the method of injection were similar to those mentioned in Experiment A, except that with each injection 1 cc. of the immune serum was given subcutaneously.

Three rabbits (Nos. 11, 12, and 13) were tested for the presence of normal agglutinins for the dysentery bacilli on Oct. 4, 1917, a procedure which was part of the routine of all experiments. None having been detected, the animals were injected shortly afterwards, as already indicated. In none of the rabbits was there any local reaction. Agglutinins were produced in all of them. As a rule the serums agglutinated Flexner bacilli in 1:50, but not Shiga bacilli, 5 days after the first injection. 5 days after the second injection the titer rose, as a rule, to 1:200 for Flexner and 1:100 for Shiga cultures. 7 days after the last injection the titer was 1:200 for both type cultures. 1 month after the last injection Rabbit 11 showed 1:50 Flexner and Shiga agglutinations, Rabbit 12, 1:200 Flexner and Shiga agglutinations, and Rabbit 13 died of an intercurrent infection (coccidiosis) before the end of that period. Rabbit 11 showed resistance to four lethal doses of a Flexner culture, although controls died in 18 and 24 hours respectively, but Rabbit 12 succumbed to ten lethal doses of a Shiga culture.¹⁹

The deduction from this series is that immune serum exerts a neutralizing effect upon the toxicity of the cultures, permitting the production of agglutinins in an appreciable amount and giving rise to partial protection, enduring at least a month after the last injection.

Gibson,⁹ to whom reference has already been made, attempted the removal of the antibacterial constituents from immune serum and the retention of the antiendotoxic substances. The method employed was the complete absorption by means of bacterial suspension of all agglutinins and other antibacterial antibodies present in polyvalent antidysenteric serum. This modified serum was injected with killed cultures of dysentery bacilli. In the next experiment an attempt was made to investigate the merits of this form of vaccination.

¹⁹ Four lethal doses of a Flexner culture were used in these tests for protection for the following reason. Our stock culture was lethal in a dose of one-quarter of a culture (the 24 hour growth on an agar slant). More than four times this quantity would prove too great an amount of bacterial protein. Ten lethal doses of a Shiga culture could be easily employed, as the total amount equalled only one-third of the growth on a standard agar slant. However, in all cases control rabbits were used to test the corresponding doses.

Experiment F.—Polyvalent antidysenteric serum of high titer was absorbed twice with Shiga and Flexner cultures by the method described by Gibson. Rabbit 14 was injected subcutaneously with 1, 2, and 3 billion killed bacteria at 5 day intervals. 1 cc. of the modified serum was injected with each dose. Considerable local reaction resulted from each injection. The rabbit showed agglutinins only after the second injection. The titer was low, 1:50 for both types of bacilli. 1 month after the last injection this rabbit survived four lethal doses of a Flexner culture, injected intravenously. Rabbit 15 was injected in the same way as Rabbit 14, except that the modified serum was mixed with the killed bacteria previous to injection. There was no local reaction. The agglutinin titer was practically the same as in Rabbit 14. 1 month after the last injection this animal succumbed to ten lethal doses of a Shiga culture.

In this series the results with regard to the protection afforded correspond exactly with those in which the whole or unmodified immune serum was employed. Gibson advises the simultaneous employment of vaccine and modified serum, each injected separately. This has not been, in our limited experience, as efficient as the combined whole serum and vaccine as practised by Lucas and Amoss.⁸

The outcome of this series of studies is the conclusion that immune serum added to the killed bacilli, or vaccine, is capable of neutralizing the toxicity of the bacilli, of stimulating the production of agglutinins, and yet of affording only partial protection. All vaccines of this kind may lead, however, to sensitization to horse serum, which is objectionable with respect to large bodies of men.

Oily Suspensions of Dysentery Bacilli as Vaccines.

The use of oils as vehicles for drugs for subcutaneous or intramuscular injection has long been practised. The principle involved is the slow absorption of the drug, unchanged in any way in the preparation, thus allowing the body to accustom itself gradually to the effects of the drug. The method has recently been applied to bacterial vaccines. Le Moignic and Pinoy²⁰ and Tribondeau²¹ suspended typhoid bacilli and subsequently typhoid and paratyphoid A and B bacilli, in certain vegetable oils, the exact nature of which has not been revealed.

²⁰ Le Moignic and Pinoy, *Compt. rend. Soc. biol.*, 1916, lxxix, 201, 352.

²¹ Tribondeau, L., *Compt. rend. Soc. biol.*, 1917, lxxx, 782.

When our studies had reached this point, the *lipo-vaccin* of Le Moignic and Pinoy was brought to our attention by Dr. Carrel. Our first tests with the oily vehicle were made during the summer of 1917. We filtered the French oily vaccine through a Berkefeld candle and thus obtained the oil free from the bacilli. Dysentery bacilli were then suspended in the oil in a proportion such that 1 cc. contained 4 billion organisms.

Experiment G.—Rabbits 16 and 17 were injected subcutaneously, under the shaven skin of the abdomen, with the vaccine in three doses of 0.25, 0.5, and 0.75 cc., corresponding to 1, 2, and 3 billion bacteria. These injections were made at 5 day intervals. No local or systemic reaction was noted. The agglutinin production was later in appearance but was more persistent than with the vaccines of the former experiments. 5 days after the first injection only a trace of agglutinins was demonstrable; 5 days after the second injection the titer was 1:100 for Flexner and Shiga types; 7 days after the third injection the titer was the same, but 1 month later it rose to 1:200 for each type. At this time Rabbit 16 resisted ten fatal doses of a Shiga culture injected intravenously, and Rabbit 17 four fatal doses of a Flexner culture. The controls died in 1 to 2 days.

From these tests it was concluded that the oily suspension answers the requirements of a serviceable vaccine: no local or systemic toxicity is caused by it; agglutinins are formed regularly in good quantities and persist; protection is secured and is still present 1 month after the vaccination.

The exact nature of the oils in the French vaccine being unknown, an attempt was made to substitute a preparation as similar as possible to that in the oily vaccine. Considerable experimentation was necessary to find a substitute. Olive oil was tested, saturated with lanolin to reduce still more the rate of absorption.

Experiment H.—Rabbits 18, 19, and 20 were injected, in a manner similar to that described in the foregoing experiment, with the olive oil vaccine. Extensive swellings developed in all three animals at the site of injection. These subsequently gave rise to large cystic masses containing on section cheesy matter (soaps). Nevertheless there was no systemic toxicity—all three rabbits withstood the injections. The agglutinin production was considerable: 1 month after the last injection the titer was 1:400 for the Flexner and 1:200 for the Shiga type. Rabbit 18 survived ten fatal doses of a Shiga culture at this time.

The olive oil was implicated in the production of the local irritation, possibly on account of the presence of oleic acid. Archard and

Foix²² experienced similar difficulties and ascribed the local abscess formation to the heating of the olive oil at too high a temperature during sterilization. With respect to agglutinin production and protection this oily vaccine was promising, but the severity of the local reaction precluded its use. Almond oil saturated with lanolin was selected for the next experiment. The results with it were more satisfactory.

Experiment I.—Rabbits 21, 22, and 23 were injected with the lanolin-almond oil vaccine in a manner similar to that of Experiment G. The first injections were not attended by any local reactions; *i.e.*, when 0.25 cc. was employed. Subsequent injections of 0.5 cc. or more, however, produced extensive indurated areas at the site of injection. The agglutinin production was marked: 1 month after the last injection the titer was 1:400 for the Flexner, 1:200 for the Shiga type. At this time Rabbit 22 survived ten fatal doses of a Shiga culture and Rabbit 23 four fatal doses of a Flexner culture, injected intravenously. Four control rabbits similarly injected died within 24 hours.

In spite of the promising results from the immunological standpoint, the outcome was unsatisfactory because of the local induration sometimes produced. The almond oil was therefore tested alone.

Experiment J.—Rabbits 24, 25, and 26 were injected in the usual manner. Neither local nor systemic reactions resulted. The agglutinin production, as a rule, for these rabbits had the following titers: 5 days after the first injection, negative for Flexner and Shiga types; 5 days after the second injection, 1:50 for Flexner and Shiga types; 5 days after the third injection, 1:100 for both types; and 1 month after the third, 1:200. Subsequent experiments proved that protection to ten fatal doses of a Shiga culture and four fatal doses of a Flexner culture, injected intravenously, was present at this time.

In view of the absence of systemic reaction and the low degree of local effect, while agglutinin production is stimulated and protection afforded, we may regard the oily vaccine as a preparation possibly suited for use in man.

Whitmore and his coworkers^{23,24} have recently published their results of the study of oily vaccines of the dysentery bacilli and some

²² Archard, C., and Foix, C., *Compt. rend. Soc. biol.*, 1916, lxxix, 209.

²³ Whitmore, E. R., Fennel, E. A., and Petersen, W. F., *J. Am. Med. Assn.*, 1918, lxx, 427.

²⁴ Whitmore, E. R., and Fennel, E. A., *J. Am. Med. Assn.*, 1918, lxx, 902.

other microorganisms. Their experiments cover a considerable number of points, including the immunizing effects on rabbits and man and the methods of preparation of the vaccine on a large scale. They employ dried cultures emulsified in a mixture of oil (olive, etc.) and lanolin by grinding in a ball mill, using glass bottles and steel balls.

Immunization by Means of a Single Injection of Oily Vaccine.

The next step in this study was the determination of dosage.

Experiment K.—Several rabbits were injected subcutaneously with a single dose of 1 cc. of plain almond oil vaccine, containing 4 billion dysentery bacilli. Rabbits 27 and 28 were carefully studied with reference to agglutinin production and the following results of slow absorption observed: 8 days after the injection the titer was 1: 50 for Flexner and Shiga bacilli; 1 month after the injection, having fluctuated within this period from 1: 50 to 1: 200, the titer was 1: 100 for either type. Rabbit 28 survived ten fatal doses of a Shiga culture, and Rabbit 27 four fatal doses of a Flexner culture, injected intravenously. Two control rabbits, injected similarly, died within 2 days.

The deduction from these tests is that a single dose of sufficient quantity of the oily vaccine is capable of yielding agglutinins for and providing protection against the dysentery bacilli which endure practically undiminished for at least a month. The experiment was next performed on a monkey.

Experiment L.—0.5 cc. of the plain almond oil vaccine containing 2 billion dysentery bacilli was injected subcutaneously into a monkey (*Macacus rhesus*) weighing 5,050 gm. After 48 hours a slight induration developed at the site of injection, but there was no active inflammatory reaction—no redness or tenderness. The monkey was lively and had no fever. The slight induration persisted for about a week and was then completely absorbed. 7 days after the injection the blood showed agglutinins for both types of dysentery bacilli: for Shiga cultures 1: 400, for Flexner 1: 200.

The study had now progressed to the point where the vaccine was ready to test on man. Several men, physicians chiefly, volunteered. Of the eight volunteers four served to determine the proper dosage and the other four to confirm the results obtained with the first group and to show the practicability of the vaccination method. The results with each individual follow.

F. G. S. was injected subcutaneously with 1 cc. of the oily vaccine, containing 5 billion bacteria. The systemic reaction was slight, consisting of headache, slight chilliness, but no rise in temperature, and lasted for 1 day. The local reaction, however, was severe. There were redness, tenderness, and induration over an area 6 cm. in diameter. This persisted for 1 week, after which absorption became evident; the entire indurated area disappeared in from 3 to 4 weeks. The agglutinin production was as follows: up to the 8th day, negative; on the 8th day, 1: 50 Flexner, 1: 25 Shiga; on the 15th day, 1: 400 Flexner, 1: 100 Shiga; 1 month later, 1: 50 Flexner, 1: 25 Shiga.

J. W. S. was injected similarly. His reactions were slightly more severe than those of F. G. S. The agglutinin production was as follows: up to the 8th day, negative; on the 8th day, 1: 200 Flexner and Shiga; on the 15th day, 1: 800 Flexner and 1: 400 Shiga; 1 month after the injection, 1: 200 Flexner and Shiga.

Hence, in as far as these two tests are concerned, the local reaction may be regarded as too severe to permit the wide use of the vaccine. However, neither of these men lost a day from his work.

On further study, however, it was found that the oil used in the preparation of the vaccine was incompletely neutralized. Hence the next two men were injected with a neutral oily vaccine, to be described later, and at the same time the dosage was diminished to 0.75 cc. containing 3,750,000,000 dysentery bacilli.

G. E. M. showed only a slight systemic reaction. There was no local reaction, except that 6 days after the injection there were slight tenderness and redness which persisted for 3 days and then disappeared. The vaccine was completely absorbed in 9 days. The agglutinin production was as follows: up to the 14th day, negative; on the 14th day after injection, 1: 50 Flexner and Shiga; 1 month after the injection, 1: 25 Flexner and Shiga.

L. E. M. had no local or systemic reaction. The agglutinin production was: on the 7th day after injection, 1: 200 Flexner and Shiga; on the 14th day, 1: 200 Shiga and 1: 100 Flexner; on the 22nd day, 1: 400 Shiga and 1: 100 Flexner; 6 weeks after the injection, 1: 400 Shiga, 1: 50 Flexner.

On the basis of these tests we concluded that 0.5 cc., containing a total of $2\frac{1}{2}$ billion bacilli (equal parts of Shiga and Flexner strains), would be a practicable dose. The following four men were given his amount.

B. G. had no systemic reaction. Locally there was an indurated area 2 cm. in diameter, which persisted for 2 weeks. It was tolerable and was accompanied by no troublesome symptom. This man is subject to boils, a fact which

may explain a slight infection which developed in the area. The complication was neither painful nor troublesome. The agglutinin production was: on the 7th day after injection, 1: 100 Flexner, 1: 25 Shiga; on the 14th day, 1: 50 Flexner and Shiga; on the 22nd day, no agglutinins for Flexner or Shiga bacilli.

F. H. M. showed a slight systemic reaction. The local reaction was shown by slight redness and moderate induration, which was absorbed in 14 days and was tolerable. The agglutinin production was as follows: on the 7th day after the injection, 1: 50 Flexner and Shiga; on the 14th day, 1: 50 Flexner, 1: 200 Shiga; 1 month after the injection, 1: 50 Flexner and 1: 400 Shiga.

D. R. B. had no systemic reaction. There was an indurated area 4 cm. in diameter, but it was not in any way troublesome and was completely absorbed in 3 weeks. The agglutinin production was as follows: on the 7th day after injection, 1: 50 Flexner, 1: 25 Shiga; on the 14th day, 1: 100 Flexner, 1: 50 Shiga; 1 month after injection, 1: 100 for Flexner and Shiga bacilli.

P. K. O. showed a slight systemic reaction. Induration developed at the site of injection, and there was tenderness of this area for 2 days. The induration was absorbed after 10 days. The agglutinin production was shown to be negative up to the 8th day; on the 18th day after injection it was 1: 200 for Shiga and Flexner bacilli; 1 month after injection it was 1: 100 for both types; 3 months after injection it was 1: 80 for Flexner and 1: 40 for Shiga bacilli.

From a summary of the experiments with the plain neutralized almond oil vaccine in man, it appears that a single injection results in slight or no systemic reaction and a moderate local reaction. The latter consists in the formation of a subcutaneous indurated area, corresponding to the unchanged oil and bacteria, which gradually recedes, 1 to 3 weeks being required for complete disappearance, during which period no inconvenience is suffered. The agglutinin production is indicative of the slow absorption of the antigen. Agglutinins tend to appear after 7 days, to increase as a rule from this time to the 3rd week, and then to persist for a month at least. In one instance an appreciable amount of agglutinin was still present 3 months after the single injection of the oily vaccine.

Mode of Action.

The advantages of the oily suspensions of dysentery bacilli for purposes of active immunization or vaccination depend on several properties. In the final analysis the desired result is achieved by the disintegration and absorption of the contained bacilli. In this respect there is no distinction between the saline and oily suspensions. The chief differences are in the rate of absorption.

In the case of the saline suspensions, disintegration rapidly ensues. The tissues at the point of inoculation are brought under the influence of the concentrated toxic products, and a sharp local reaction follows. The rapid absorption of these products sets up also a marked general reaction.

On the other hand, the suspended bacilli are only slowly yielded up by the oil; hence disintegration is gradual and the local toxic action on the tissues minimized. Moreover, and for the same reason, absorption proceeds slowly, so that the general reaction is either eliminated altogether or greatly diminished; and yet the immunity response is at least as great in the latter instance as in the former, and it may even be greater, since it occurs with less cost to the organism of the host as a whole.

The rate of absorption of the two kinds of suspensions was studied experimentally.

Experiment M.—Rabbit 29. Nov. 14, 1917. Injected subcutaneously with 1 cc. (equals 2 billion dysentery bacilli) of saline suspension vaccine. Nov. 15. Swelling and redness over an area of 5 by 3 cm. The area was aspirated and the drop of fluid obtained examined in a film preparation stained by Gram's method. No dysentery bacilli were seen. There were present a few polymorphonuclear cells, most of them fragmented, and a number of large endothelial cells. Nov. 16. The appearances in the film preparations of the exudate were identical with those of the day before. Nov. 17. No exudate obtainable.

Rabbit 30. Nov. 14, 1917. Injected subcutaneously with 1 cc. of almond oil vaccine (equals 2 billion dysentery bacilli). Nov. 15. 0.25 cc. aspirated from the indurated area caused by the injection. The aspirated fluid was grossly indistinguishable from the oily vaccine. Microscopically, there were numerous Gram-negative bacilli, a few large flat endothelial cells, and a few polymorphonuclears containing numerous bacilli (phagocytosis). Nov. 16. The aspirated fluid showed no change. Nov. 17. The aspirated fluid showed only a trace of oil and a few Gram-negative bacilli and fragmented polymorphonuclears. The bacilli showed lytic changes. Nov. 18. No exudate obtainable.

From this experiment one may conclude that with the saline suspension the bacteria disappear from the site of injection in 24 hours, with the oily vaccine not until after the 3rd day. In the mode of operation of the latter, therefore, slow absorption is the essential factor.

The Shiga bacillus is capable of developing in culture media, besides an endotoxin, a soluble toxin of great potency.^{22, 25, 26} The efficacy of the oil in mitigating the effect of this toxin is shown by the following experiment.

Experiment N.—By growing a toxic strain of the Shiga bacillus in a medium of neutral sugar-free broth to which a protein, such as egg albumin, is added, a toxin is obtained the minimum lethal dose of which varies between 0.05 and 0.1 cc. The sample, Toxin 4, used in the following test was prepared Dec. 1, 1917, and when injected intravenously into a rabbit in a dose of 0.1 cc. caused paralysis of the posterior extremities after 3 days and death on the 4th day. Autopsy revealed the typical intestinal lesions of Shiga bacillus inoculation of the rabbit. The toxin resists drying.

Rabbit 31. Jan. 2, 1918. Injected with ten minimum lethal doses of dried (*in vacuo*) Toxin 4 emulsified in 1 cc. of saline solution and kept at 37°C. for 30 minutes. Jan. 3. Paralysis of posterior extremities; diarrhea. Jan. 4. Found dead.

Rabbit 32. Jan. 2, 1918. Injected intravenously with ten minimum lethal doses of the dried Toxin 4 emulsified in 1 cc. of plain almond oil and incubated at 37°C. for 30 minutes. The animal survived, with no loss of weight or other symptoms. The experiment was repeated with twenty minimum lethal doses of dried Toxin 4 with similar results: the dried toxin in saline solution caused the death of Rabbit 33 in 2 days; the dried toxin in the oil gave rise to no symptoms in Rabbit 34.

Owing to the presence of the oil, the toxin is probably absorbed in subliminal amounts.

Development of the Shiga Antitoxin.

The next experiment bears on the efficacy of the oily vaccine in leading to antitoxin production.

Horses receiving injections of bacteria show the presence of antitoxin only after a considerable period of time and in small quantity. For example, a horse which had been injected with Flexner and Shiga bacilli continuously for 3½ years yielded a serum of which 0.001 cc. neutralized a minimum lethal dose of Shiga toxin, or, on the basis of 100 minimum lethal doses, 1 cc. contains ten units. The limit of neu-

²⁵ Doerr, R., *Das Dysenterietoxin*, Jena, 1907, 30 and ff. Kraus, R., and Doerr, R., *Wien. klin. Woch.*, 1905, xviii, 158.

²⁶ Todd, C., *J. Hyg.*, 1904, iv, 480.

tralization of normal horse serum is 0.5 cc. for four minimum lethal doses of Shiga toxin.

Experiment O.—*Macacus rhesus*. Nov. 7, 1917. Bled for trial titration. 2 cc. of the serum were mixed with ten minimum lethal doses of Toxin 4, incubated for 30 minutes at 37°C., and injected into Rabbit 35. The rabbit developed complete paralysis and died in about 20 hours. No antitoxin, therefore, could be demonstrated in 2 cc. of the monkey's blood. The monkey was then injected subcutaneously with 0.5 cc. (equals 2 billion dysentery bacilli) of the oily dysentery vaccine and 7 days later similarly with 1 cc. 1 month later the monkey was bled again and 0.1 cc. of serum plus ten minimum lethal doses of Toxin 4, after incubation at 37°C. for 30 minutes, were injected intravenously into Rabbit 36. The rabbit became paralyzed after 48 hours and died on the 6th day (faint trace of neutralization). 0.5 cc. of serum plus ten minimum lethal doses of Toxin 4, injected into Rabbit 37, gave rise to paralysis after 4 days and death on the 6th day (trace of neutralization). 1 cc. of serum plus ten minimum lethal doses of Toxin 4, injected into Rabbit 38, caused no symptoms (complete neutralization). Rabbit 39 (control), injected intravenously with ten minimum lethal doses of Toxin 4, died within 24 hours.

The preceding experiment confirms the fact that in the *rhesus* monkey, an animal closely related to man, antitoxin may be produced as a consequence of injecting the oily vaccine. A similar test was made with human blood.

Experiment P.—J. W. S. had been injected, 2 months before the following test was made, with a single dose of the oily vaccine.

Rabbit 40 was injected intravenously with ten minimum lethal doses of Toxin 4 plus 1 cc. of J. W. S.'s blood serum. The toxin and the serum were kept at 37°C. for 30 minutes before the injection. The animal survived. Of two control rabbits, one, injected with ten minimum lethal doses of Toxin 4, died in 48 hours; the other, injected with 1 cc. of normal human blood serum mixed with ten minimum lethal doses of the same toxin, died in 3 days, after manifesting the typical intestinal symptoms.

The significance of these experiments is clear. The Shiga bacillus, which is the most difficult to employ for vaccination in man because of its toxic effects, is unimpaired in its antigenic properties by the oil. Hence, although no marked reaction is set up, the immunity response is definite. Not only are antibacterial antibodies produced, but in the case of the Shiga toxin, antitoxin as well; and the latter is demonstrable 2 months, at least, after the injection.

Preparation and Administration of the Oily Vaccine.

Commercial vegetable oils, including almond oil, may contain impurities and objectionable quantities of free fatty acids. Hence, to avoid the former one should use only a rectified oil, and, in the case of almond oil, only that of sweet, not of bitter almonds. To eliminate the free fatty acids each specimen of oil should be neutralized. This may be done as follows:

The oil is dissolved in an equal volume of ether. Sodium methyrate is prepared, in the meantime, by dissolving 20 gm. of metallic sodium in 100 cc. of dry (absolute) methyl alcohol. The fresh sodium methyrate (it will not keep for more than a week) is added to the dissolved oil to the point of neutralization, a few drops of an alcoholic solution of phenolphthalein being used as indicator. If by mistake an excess of the alkali is added, the harm may be undone by adding more of the original oil. The next day the neutralized oil is filtered by suction or centrifuged to remove the precipitate of soaps, and the ether is evaporated by warming. It is kept in the refrigerator for 1 week, animal charcoal is added, and it is again filtered. It is then sterilized by autoclaving for 45 minutes at 15 pounds pressure.

The oily vaccine is prepared in the following manner. Plain agar cultures in Blake bottles are made of the Shiga, Flexner, and Y bacilli. 24 hour growths are washed off in normal saline solution, usually 5 cc. of saline solution to each bottle being sufficient. The suspension of each strain is thoroughly shaken and a portion set aside for counting, which is done by Wright's method. In the meantime the saline suspensions are transferred to wide centrifuge tubes, 2.5 by 10 cm., and are centrifuged for 20 minutes at about 1,800 revolutions per minute. Since the cotton stopper may be jammed to the bottom the cotton is replaced by sterile tin-foil, folded over the edge of the tube. At first, drying *in vacuo* was attempted, but it proved inconvenient, and centrifugation was substituted to obtain the bacterial mass, with good results. Almost all the saline solution can be pipetted or poured off, leaving a densely packed sediment of bacteria. The count of the bacteria and the amount of saline solution having been determined, it is a simple matter to add sufficient oil, replacing the saline solution, so that 1 cc. will contain $2\frac{1}{2}$ billion

Shiga and $1\frac{1}{4}$ billion each of Flexner and Y bacilli. The total content in 0.5 cc. of the vaccine should be $2\frac{1}{2}$ billion dysentery bacilli in aliquot parts of the Shiga and Flexner groups of organisms. The required amount of oil is added to the centrifuged bacterial mass, and by means of a bent glass rod an oily suspension is made. The suspension is then transferred to receptacles containing glass beads. Glass beads must always be included, irrespective of the quantity of vaccine in a container. They are the essential factor upon which a proper suspension depends. No preservative is added, as it has been determined that it is without action in oils. The oily vaccine is heated to 60°C . for $\frac{1}{2}$ hour. Tests are made for sterility and for its effect in the rabbit. The vaccine is stored in the refrigerator.

The oily vaccine is warmed slightly before use, in order to make it more fluid, and shaken thoroughly. The skin over the deltoid region is cleansed with alcohol, tincture of iodine is painted on it, and 0.5 cc. of the vaccine is injected subcutaneously. Care is taken to avoid intracutaneous or intravenous injection.

DISCUSSION AND SUMMARY.

The purpose of the present investigation was to determine a practical method of vaccination against bacillary dysentery.

It has been emphasized that the toxicity of the dysentery group of organisms, especially that of the Shiga bacillus, is such as not to permit of their employment in simple saline or aqueous suspensions. If, on the other hand, their toxicity is removed by the addition of certain chemicals, the antigen of the bacilli is so changed as not to be suitable for immunization purposes.

The toxicity of the bacilli can also be diminished by the addition of immune serum. There is no essential difference in the result whether unmodified serum is used or that modified by Gibson's method. The use of serum with vaccines cannot be recommended, in spite of the relative non-toxicity of such mixtures. The specific immunity response is reduced, while the parenteral injection of horse serum in large groups of men is objectionable because of the serum sensitization which it produces.

It has been shown that a certain vegetable oil, almond oil, (and this statement may apply to a number of non-irritating, absorbable oils) is capable of overcoming many of these disadvantages.

The oil acts as a passive agent in merely suspending the bacteria without altering their properties. The slow absorption of the suspended bacteria from this vehicle mitigates the toxic effects of the dysentery bacilli. At the same time it does not interfere with the immunity response—antibacterial and antitoxic.

If the absorption is too slow, however, as in the instances in which lanolin was added, less satisfactory results follow. The proper rate of absorption is as important a factor as the proper vegetable oil. The latter condition has been emphasized in the consideration of the effects of an objectionable olive oil.

The neutralization of the oil should be complete. F. G. S. and J. W. S. suffered from severe local reactions, the results of the local deposition of soaps, which are difficult of absorption.

As a result of the slow absorption of the dysentery bacilli from the oily suspension only slight local and general reactions follow, and it is possible to give at one time and in a single dose a sufficient number of the killed dysentery bacilli to incite a high degree of immunity.

The precise series of events following the injection of the oily vaccine are: During the 1st day, an erythematous area develops at the site of injection, which is not especially painful. There may be a slight systemic reaction, consisting in headache and slight chilliness. After 24, sometimes after 48 hours, an induration appears at the site of injection, varying from 2 to 4 cm. in diameter, which remains unchanged for a few days, then begins to recede, complete absorption requiring from 1 to 3 weeks. The induration remains localized and has no tendency to break down. It causes no inconvenience.

During the period of absorption the organism of the host continually receives antigen. The rule of immunology is that while antigen is circulating the antibodies are usually not demonstrable; it is only after all the antigen disappears that the antibodies appear in greatest concentration. Since the vaccine is slowly absorbed, the reasons for the delayed appearance of antibodies, as well as their persistence, become obvious.

CONCLUSIONS.

The preceding study seems to emphasize the advantages of a bland oily medium for suspending dysentery bacilli for purposes of active immunization or vaccination. The experiments on animals and a small number of tests on man indicate that the single injection of an almond oil suspension of the Shiga and Flexner groups of dysentery bacilli suffices to afford protection as indicated by the appearance in the blood of definite specific antibodies for each group of the bacilli, and by the protection of animals from otherwise lethal doses of the living organisms or their toxic products. The extent to which vaccination should be applied to man will depend on circumstances and conditions still to be defined, but the method appears to be wholly practicable. The introduction by Le Moignic and Pinoy of the oily medium for suspending killed bacteria for immunization purposes marks a definite advance in the technique of bacterial vaccination.

EXPERIMENTAL TRYPANOSOMIASIS: ITS APPLICATION IN CHEMOTHERAPEUTIC INVESTIGATIONS.

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PLATES 4 TO 16.

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Although experimental trypanosomiasis has been the subject of countless contributions to the literature, comparatively little has been written regarding the biological principles involved in the adaptation of the experimental infection to chemotherapeutic investigations. There have been numerous reports dealing with the more or less successful treatment of experimental trypanosomiasis beginning with Laveran and Mesnil (1), who showed in 1902 that arsenite of soda had a marked although only a temporary beneficial effect on the course of nagana infections in mice. 2 years later Ehrlich and Shiga (2) published their remarkable results with trypan red with which they were able to cure mice infected with caderas. This contribution marked the beginning of a systematic search for synthetic compounds which would exercise a specific therapeutic effect upon this class of infections, but despite the extensive investigations that have been directed toward this end during the past 15 years, the basic concepts underlying the adaptation of experimental trypanosomiasis to chemotherapeutic investigations have remained rather obscure. The experimental procedure employed by different investigators has been of the most diverse character, yet little has come out of these years of experience that would aid one either to a more judicious use of the material at his disposal or in the correct interpretation of therapeutic results obtained. The available information, such as it is, is so diffusely scattered through the literature that it is a well-nigh impossible task for one not thoroughly conversant with the facts to assemble it or to discriminate among the bewildering mass of statistics, reports, inferences, and statements with which he is at once confronted. In

view of this situation, we have attempted to present some of the salient features of experimental trypanosomiasis together with an analysis of the biological factors underlying its adaptation to chemotherapeutic investigations.

Trypanosomiasis in Nature.

Trypanosomiasis comprises a group of widespread infectious conditions, some form of which is found in practically all parts of the world. Both warm and cold blooded animals are naturally susceptible to infection with one or another species of the organism. In some instances, it is an apparently harmless parasitic condition, while in others, the infection produces grave manifestations of disease. For medical and economic reasons, the greatest interest has naturally been directed toward those forms of the disease affecting man and the domestic animals. Chief among these are the human infections with *Tr. gambiense* and *Tr. rhodesiense* which cause the so called sleeping sickness of Africa, and the animal infections due to *Tr. brucei*, *Tr. evansi*, *Tr. equiperdum*, *Tr. equinum*, *Tr. dimorphon*, *Tr. theileri*, and *Tr. congolense*. Of these organisms, *Tr. brucei*, which causes the nagana of wild and domestic animals of Africa, *Tr. dimorphon*, known as the causative agent of Gambian horse disease, producing a chronic infection in other domestic animals as well, *Tr. theileri*, the causative agent of galzielte or bile fever in cattle, and *Tr. congolense*, which produces a chronic infection in horses, cattle, sheep, and dromedaries, are all confined to Africa. *Tr. evansi*, the causative agent of surra, is much more widely distributed, being found throughout Southern Asia and the adjacent islands, as well as Northern Africa. The mal de caderas in horses produced by *Tr. equinum* is confined to certain sections of South America, while dourine of horses produced by *Tr. equiperdum* is widely distributed through Africa, Asia, and certain portions of the United States, Canada, and Southern Europe.

As a class, these diseases both of man and of animals are characterized by general constitutional disturbances such as febrile periods during which the infecting organisms may be present in the blood, by anemias, emaciation, and weakness, and by general or localized edema together with a variable degree of localized inflammatory reaction

and granulomatous and ulcerative lesions. In most of these diseases, the mortality is extremely high, in some uniformly fatal, while in others, the course of the infection is relatively mild.

In general, the trypanosomes causing these diseases can be successfully inoculated into such animals as mice, rats, guinea pigs, rabbits, cats, dogs, monkeys, sheep, goats, pigs, horses, donkeys, mules, and cattle, thus affording a great variety of experimental infections capable of endless propagation. The ease with which these experimental infections may be produced is extremely variable as is the character of the resulting infections which may vary from an acutely fatal infection of a few days' duration in mice to marked chronic infection of many months' duration in the larger animals.

Experimental Infections Employed by Other Investigators.

During recent years, many of these experimental infections have found wide application in the chemotherapy of experimental trypanosomiasis. Experimental nagana has perhaps been most frequently employed for such purposes. The French and German investigators have used mice for most of their experiments, supplemented in many instances by larger animals, while many of the English workers have selected the rat as the animal of choice for all routine work.

Ehrlich and Shiga (2) used mice infected with nagana and caderas for the majority of their experiments. Roehl (3), working with atoxyl and arsenophenylglycine in Ehrlich's laboratory, used mice infected with the "ferrox" strain of nagana for most of his experiments, but carried out some experiments also on rabbits and guinea pigs infected with nagana and on rabbits infected with dourine. Schilling (4), also working with arsenophenylglycine, used nagana infections in mice, rats, dogs, and horses, but again the bulk of the work was done with mice. Browning (5) used mice infected with nagana and with dourine in testing arsacatin, and Morgenroth and Halberstädter (6) used nagana mice.

Laveran and Mesnil (1) in their first chemotherapeutic experiments worked with nagana mice. Nicolle and Mesnil (7) in their work with the benzidine derivatives used mice infected with *Tr. brucei*, *Tr. evansi*, and *Tr. equinum*. Laveran (8) in his experiments with arsenious acid and trypan red used mice, rats, dogs, and monkeys and in working with atoxyl, arsacatin, arsenic trisulfide, and tartar emetic, used guinea pigs infected with *Tr. dimorphon* and *Tr. congolense*. More recently, Laveran and Roudsky (9) in testing the activity of galyl used eight strains of trypanosomes in mice and but one in guinea pigs. Lafont and Dupont (10) used rats in their work with galyl and ludyl. In most of the experiments

with his new synthetic compounds 88² and 102¹ (luargol), Danysz (11) used mice infected with *Tr. evansi*, *Tr. rhodesiense*, *Tr. gambiense*, and *Tr. dimorphon*, although he also reports some experiments with surra rabbits. Yakimoff and Wassilevsky (12) selected dourine mice for their experiments with luargol.

The English investigators, Breinl and Nierenstein (13), recommend the rat as the animal of choice, stating that the most conclusive evidence can be obtained with this animal. Their experiments were carried out with infections produced by *Tr. brucei*, *Tr. equiperdum*, and *Tr. gambiense* in rats, guinea pigs, donkeys, and monkeys. Plimmer and Thomson (14) selected rats infected with nagana and surra for their work with antimony compounds. Wenyon (15) conducted a long series of experiments with benzidine dyes on mice infected with *Tr. dimorphon*, and Seidelin (16) tested the therapeutic activity of salvarsan-copper in rats infected with *Tr. brucei*. On the other hand, in contrast to the somewhat limited procedure of many workers, Thomas and Breinl (17) in their therapeutic experiments with atoxyl and trypan red employed a considerable number of trypanosomal infections (*Tr. gambiense*, *Tr. evansi*, *Tr. brucei*, *Tr. equinum*, *Tr. equiperdum*, and *Tr. dimorphon*) in practically all the common laboratory animals and contrary to general practice, their conclusions regarding the efficacy of these drugs were based for the most part on the results obtained in the larger animals.

In American laboratories, comparatively little has been done on the chemotherapy of experimental trypanosomiasis. Rowntree and Abel (18), working with compounds of antimony, used *Tr. brucei*, *Tr. evansi*, and *Tr. dimorphon* infections in rats, rabbits, dogs, and one donkey, while Schamberg, Kolmer, and Raiziss (19) have expressed a preference for rats infected with *Tr. equiperdum*.

These examples will suffice to show something of the way in which different forms of experimental trypanosomiasis have been used in chemotherapeutic investigations. Doubtless each worker has had his own reasons for using one or another of these experimental infections, but, with few exceptions, little space has been devoted to the exposition of these reasons. The definite conception of the clinical significance of experimental infections in larger animals held by Thomas (20) as the basis for deductions from his therapeutic experiments with atoxyl, raised for the first time the importance of these factors in the ultimate determination of therapeutic efficiency. Later Breinl and Nierenstein (13) devoted considerable space to a discussion of the relative merits of the use of rats in chemotherapeutic experiments and expressed the opinion that the most conclusive evidence can be obtained with rats as they are very susceptible to infections, their reaction is very constant, and their relapsing time is fairly regular and in direct proportion to the trypanocidal action of different com-

pounds. As regards the other animals, they cited the facts that guinea pigs often died after a short course of treatment from no apparent reason and that in the chronic infection of rabbits, parasites were usually present in exceedingly small numbers and the clinical symptoms were generally easily controlled by the trypanocidal drugs. Further, since mice tolerate, in proportion to the body weight, immense doses of different drugs, they considered it unwise to draw general conclusions from mice experiments for the use of drugs in the treatment of sleeping sickness and animal trypanosomiasis. Finally, Uhlenhuth, Hübener, and Woithe (21) pointed out the difference between such infections as those of mice and rats, in which the disease is largely a blood infection, and that of rabbits, in which tissue involvement is the conspicuous feature, but apparently made little use of this fundamental principle.

Taken as a whole, mice have been much more extensively used than any other animal and the infecting organism most commonly employed has been *Tr. brucei*. Next in frequency have come rats, while guinea pigs, rabbits, and other animals have been used in small numbers and largely to supplement mouse or rat experiments. Much the same condition has prevailed as regards the use of different species of trypanosomes. As the matter now stands, this diversity of usage is very disconcerting and might seem to indicate that there is very little involved in the question of the adaptation of experimental trypanosomiasis to chemotherapeutic investigations and that it mattered but little which animal or which organisms were used for the experiments or how they were used. As a matter of fact, this is far from the case and we are inclined to interpret this lack of any concerted plan of procedure as evidence of a too restricted view of the factors involved and, in addition, as a need for a more critical analysis of these factors as a basis for the adaptation of the experimental conditions to the requirements of the investigator. That there are definite requirements to be met may be self-evident, but nowhere do we find a very clear statement of what they may be. Reduced to the simplest terms, the essential requirements of the worker in chemotherapy of trypanosomiasis are a means of determining quickly and accurately the activity of substances upon an infecting organism and a means of determining the curative powers of these substances, which, as will

appear later, may be problems of an entirely different character. The solution of these problems can best be approached through a careful analysis of the various forms of trypanosomiasis including both animals and organisms that may be used, the means of propagating and regulating the infection, and finally, the general response of various species of trypanosomes to therapeutic agents.

Experimental Trypanosomiasis.

Propagation of the Infection.

The propagation of experimental trypanosomiasis may be accomplished in many ways. In principle, the blood of an infected animal which contains the organisms is used as the medium of transference of the infection from animal to animal. This blood, drawn either from a peripheral vessel or from the heart, is diluted with a suitable medium such as isotonic salt solution or a dilute sodium citrate solution and the requisite dose of the infecting suspension is introduced into the next animal, either subcutaneously, intraperitoneally, or intravenously. The general scheme of animal inoculation may be illustrated by a brief description of the methods which we have employed for several years.

For reasons which will appear later, it is advisable to maintain the stock organisms in two classes of animals, one in which the infection is an acute progressive blood infection as in the mouse or the rat, and the other in which the infection pursues a more chronic course such as the guinea pig. Continuous direct passage from animal to animal of the same species is desirable in order to maintain uniformity of infection reactions where the organisms are to be used for therapeutic experiments. In transferring stock strains of trypanosomes, the blood from a guinea pig showing a well developed infection (microscopic examination of the blood) with actively motile and non-agglomerated trypanosomes is taken from the ear vein or heart with sterile precautions and diluted with sterile normal salt solution until one sees only one trypanosome in every five or six microscopic fields using the high dry objective. We arbitrarily designate such a suspension as +. Varying amounts of a + suspension (1, 0.5, 0.25, and 0.1 cc.) are injected intraperitoneally into guinea pigs. The precaution is

taken of inoculating several stock guinea pigs at the same time with different sized doses in order that the animals may not all die at approximately the same time and so make the recovery of the strain uncertain, and furthermore, since there is considerable irregularity in finding parasites in the peripheral blood of guinea pigs, it is convenient to have on hand stock animals with varying grades of infection so that there may be as little delay as possible when one wishes to infect series of animals for experimental purposes.

Stock mice are inoculated intraperitoneally with a similar + suspension of infected blood in sterile normal salt solution. The tail of the infected mouse, one showing a + + + or + + + + infection, is cleansed with alcohol and dried and is then bled directly into a small Petri dish containing sterile normal saline solution. On account of the variation in virulence of different species of trypanosomes, we use various sized inoculating doses in order to maintain comparable grades of infections. With our particular strains, the doses used for mice are 0.2 cc. of a + suspension of *Tr. brucei*, *Tr. equiperdum*, and *Tr. evansi* and 0.5 cc. of a similar suspension of *Tr. gambiense* and *Tr. equinum*.

Since it is not practicable to maintain stock strains of trypanosomes in all species of animals which may be used for therapeutic purposes, the proper source of the virus to be used in the inoculation of any given animal may be determined, in general, upon the basis of analogy of infection types; that is, the animals which show an acute, progressive, blood infection should be inoculated, if practicable, from a stock virus in which an infection of the same general type is maintained, and, conversely, animals showing a chronic, cyclic infection should be inoculated from a stock virus where a like infection has been maintained. (The influence of passage upon the general type of infection produced, as well as upon the virulence of the organism in question, will be discussed at greater length in subsequent sections of this paper.) To meet these conditions, mice or rats to be used for therapeutic experiments should be inoculated with trypanosomes from a stock strain carried in mice or rats, while guinea pigs and rabbits may be inoculated from a guinea pig stock. The inoculation of such animals is carried out in the following way.

In making subinoculations from stock mice to series of mice and rats for therapy experiments, a + suspension is prepared in the same

way as for stock inoculation and the same sized inoculating dose is used. If a large series of animals is required, it is convenient to anesthetize the stock mouse and bleed directly from the heart into sterile salt solution. When inoculating experimental series of guinea pigs and rabbits, it is our custom to bleed from the heart, using a stock guinea pig which shows a +++ or ++++ infection, and if a large number of animals are to be inoculated, it is a wise precaution to keep the blood suspension slightly warm. We prefer guinea pigs of 350 to 500 gm. weight for therapeutic experiments and inoculate them intraperitoneally with 0.5 cc. of a + suspension of *Tr. brucei*, *Tr. equiperdum*, and *Tr. evansi* and with 1 cc. of *Tr. gambiense* and *Tr. equinum*. Rabbits are infected intravenously with 1 cc. per kilo of body weight of a similar + suspension of *Tr. brucei*, *Tr. equiperdum*, and *Tr. evansi* and 1 cc. per kilo of a ++ to +++ suspension of *Tr. gambiense* and *Tr. equinum*. We have found with our strains that intraperitoneal inoculations of mice, rats, and guinea pigs, and intravenous inoculations of rabbits give fairly uniform and as a rule satisfactory results as regards the incubation period, the grade and course of the infection, and the length of life of the animal. Certain variations in these points, regardless of the uniformity of dosage, are not infrequent in the larger animals as will appear later.

Animals and Organisms.

Since we have in mice and rats on the one hand, and in guinea pigs and rabbits on the other, two groups of animals in which experimental trypanosomiasis manifests itself as two essentially different types of infection, the chemotherapist is confronted by two distinct problems in the treatment of such infection types and hence a choice of animals offers an opportunity of testing the therapeutic efficacy of a compound both as regards its trypanocidal action in the acute blood infections and its potency or curative power in the chronic tissue infections. In addition to this fundamental factor of the infection type, as exemplified by the animal species, the question of time and expense must be considered. Mice and rats are the cheapest animals, they can usually be obtained in abundance, are easily handled, and the results of treatment of these infections are quickly obtained. Com-

paring the results of treatment of infections in mice and rats where the infection is of the same general order, we have found that while there is little difference as regards ease of demonstration of therapeutic activity in the two animals, mice are usually more easily cured than rats and the results of treatment are more constant and more uniform in character both as regards the absolute unit dose and the ratio of the curative to the tolerable dose. Moreover, the end-result of treatment can be determined much more quickly in mice.

Trypanosomal infection of guinea pigs and rabbits is a chronic disease in which tissue involvement and not blood infection is the fundamental factor. The treatment of such a pathological condition is a manifestly different problem than the treatment of an acute blood infection and is more nearly analogous to the naturally acquired forms of trypanosomiasis in man and animals. However, the extensive use of guinea pigs and rabbits entails a considerable expenditure of time and money, and in our experiments we have not attempted to use them for routine preliminary tests of trypanocidal activity of a given substance but have reserved them for the more detailed study of those substances which have shown such activity against the infection in mice and rats. Other animals, such as monkeys, dogs, horses, donkeys, cattle, sheep, and goats have been used by different workers especially to simulate naturally acquired forms of the disease. The infection in all of these animals tends to be subacute or chronic with more or less tissue involvement, depending on the trypanosome used and the size of the inoculating dose, thus falling into the group to which the rabbit and guinea pig belong, with a possible exception of the dog, which when infected with nagana, for instance, may have an acute, rapidly fatal infection, with the almost constant presence of parasites in the peripheral blood.

As in the selection of animals for chemotherapeutic study, a choice of the species of trypanosomes is of equal importance. This choice should be based on the character of the infection produced in the various animals employed and the general response of the organism to therapeutic agents as far as this is known. In our work we have used five species of pathogenic trypanosomes and from the point of view of the general character of infection produced, they fall into two groups. *Tr. brucei* and *Tr. evansi* are highly virulent for laboratory

animals, producing a relatively acute, rapidly progressive, and usually fatal infection, while organisms of the second group, *Tr. equiperdum*, *Tr. gambiense*, and *Tr. equinum*, are less virulent, causing a more prolonged course of infection from a given inoculating dose which terminates fatally in mice and rabbits and usually so in rats and guinea pigs. It must be borne in mind, however, that different strains of the same species of trypanosome may show wide divergences in virulence and in the general character of the infection produced. This, in part, may be due to the length of time of isolation of the particular strain from its original source, together with the rate and number of passages in the particular species of stock animal, as well as inherent differences in virulence of individual strains of the same species. The factor of resistance or response of the various species of trypanosomes to therapeutic agents will be considered in a later section.

The general character of the infections, such as the incubation period, course, and duration of the infection, as observed by a number of investigators, is recorded in Tables I to VIII, for convenience of comparison.

A complete analysis of the data contained in these tables is impossible within the scope of this paper, but on the surface it is evident that experimental trypanosomiasis is an extremely varied condition not only as to the infections produced by different species of trypanosomes but even to the character of the infections produced by a given species of trypanosome in one and the same animal species. While the full significance of these facts to the chemotherapist can hardly be gathered from a mere statistical compilation, it may be seen that, in as far as the common laboratory animals are concerned, there is a fairly well defined line of cleavage between the acute infections produced by this group of organisms in such animals as mice and rats on the one hand, and the more chronic infections in guinea pigs and rabbits on the other. As we have already suggested, these differences are of fundamental importance, but their chemotherapeutic significance can be made fully apparent only by an elaboration of the clinical characteristics of the infections in the different animal species.

TABLE I.

Tr. brucei (Nagana).

Investigators.	Mice.			Rats.			Guinea pigs.			Rabbits.			Other animals.		
	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.
Laveran and Mesnil.	I. P. ‡	hrs. 24	3	I. P.	days 2	2½	S. C.	days 2-4	days 15-30	I. V.	days 2-3	days 10-50	S. C.	days Dogs. 2-4	days 6-14
Kanthack, Durham, and Plimmer.		days 2	13* (8-25†)		2	3½-5½	I. P.	days 2-4	days 15-30*	S. C.	days 2-3	days 10-50			
Bradford and Plimmer.			9			12*		days 5-7	days (5-61†)		8	30*	S. C.	4-6	18* (14-26†)
Plimmer and Thomson.						6			days (20-183†)			(13-58†)			
Seidelin.					2-3	5½									
Bruce (Ouganda strain).	S. C.		10-12			8-17									Dogs. 8-16
Roehl ("ferrox" strain).		2	hrs. 60-70									mos. 1-3			
Browning.	S. C.	hrs. 24	days 3												
Rowntree and Abel.						hrs. 72-84						days 12-20	I. P.	Dogs. hrs. 48-72	5-14
Thomas and Breinl.			hrs. 60-72		hrs. 36-48	days 4-5§	S. C.		days 40-45			mos. 1-3			
Pearce and Brown.	I. P.	24		I. P.		4-6	I. P.	3-7	days 14-28	I. V.	7-10 (S.)				

The references for the authors quoted in Tables I to VIII are taken from Laveran and Mesnil (Trypanosomes et trypanosomiasés, Paris, 2nd edition, 1912) and the articles specifically mentioned in the bibliography of this paper.

* Average. † Extremes. ‡ In the tables I. P. indicates intraperitoneal; S. C., subcutaneous; and I. V., intravenous. S. indicates signs; M. S., marked signs. § Estimated.

TABLE II.
Tr. evansi (Surra).

Investigators.	Mice.			Rats.			Guinea pigs.			Rabbits.			Other animals.		
	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.
Laveran and Mesnil (Mauritian strain).	S. C.	days	11½	S. C.	days	11	S. C.	days	34		days	4		days	28 (Mauritian strain). 12-13 (Indian strain). Goats and sheep. Disease lasts about 5 mos. and usually ends in recovery.
Thomas and Breinl (Mauritian strain). Danysz.	S. C.	3½-4	9½-12	S. C.	3-4½	8-11½		6-8	40-120	I. V.	3½-5½		S. C.	Dogs. 7-9	
	I. P.	3	9-11	I. P.	2¾-3½	7¾-10½					5-7	days 30-50 (M.S.)			
Plimmer and Bateman. Rowntree and Abel.	S. C.		5												14 (dog; weight 20 lbs.). 12-15 (Indian strain). 14-21 (Mauritian strain).
Pearce and Brown.	I. P.	hrs. 24-36	4-8	I. P.	hrs. 36-48	7-8	I. P.		4-7	I. V.	7-14 (S.)	3-6			

TABLE III.
Tr. equinum (Mal de Caderas).

Investigators.	Mice.			Rats.			Guinea pigs.			Rabbits.			Other animals.		
	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.
Laveran and Mesnil.	S. C.	days 2½	days 6	S. C.	days 3-4	days 7½	S. C.	days 9	days 8½* (29-120†)	S. C.	days 4-5	days 33*	Pigs, sheep, goats, and cattle contract the disease after exposure to inoculation, in a very mild form which nearly always ends in recovery.		
	I. P.	hrs. 30-40	5												
Thomas and Breinl.	Mice become infected somewhat earlier than rats and the duration of the disease is slightly shorter.			S. C.	2¼-3¼	6-8	S. C.				4-6	16-59			
				I. P.	2-2½	6-8									
Ehrlich and Shiga. Voges.		24	4-5												
	I. P.	24-48	5-8†	I. P.	hrs. 24-48	5-8†§	I. P.		wks. 4-8§	I. V.	7-14 (S.)	mos. 1-3 wks. 3			
Pearce and Brown.	I. P.	24-48	5-8†	I. P.	24-48	5-8†§	I. P.								

* Average. † Extremes. ‡ Occasionally 10-14 days. § Occasionally spontaneous recovery. || Heavy infection.

TABLE IV.
Tr. gambiense (Sleeping Sickness).

Investigators.	Mice.			Rats.			Guinea pigs.			Rabbits.			Other animals.		
	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.
Laveran and Mesnil.	S. C.	hrs.	days	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.
Monfort.			8*			10†			days	25-291		days			
Lafont and Dupont.			(3-15†)												
DeBeurmann,		24	4-5			12									
Mouneyrat, and Tanon.															
Danzsz.	S. C.		6							Wks. or mos. §		5-15			
Thomas and Breinl.												128			Dogs. Some died in 3 wks., but majority survived longer, up to 9 mos. Occasional spontaneous recovery.
															Monkeys and baboons. Very irregular length of survival, some living over a year.
Pearce and Brown.	I. P.	24-48	5-8	I. P.	3-4	5-11	I. P.		mos.	2-6¶			I. V.	7-15 (S.)	wks. 8*

* Average. † Extremes. ‡ 3 mos. with another virus. § Death occurred 4-16 wks. after the appearance of the parasites. With a chronic mild infection lived 150-273 days. ¶ Occasionally 12 mos.

TABLE V.

Tr. equiperdum (Dourine).

Investigators.	Mice.			Rats.			Guinea pigs.			Rabbits.			Other animals.		
	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.
Laveran and Mesnil (Rouget virus).		days	days		days	days			wks.		days	mos.	S. C. Vul- va.	mos. Dogs. 2½	mos. 9 2½
Rouge			6-8			15*	At first guinea pigs were refractory but later virus became virulent for them.					1-3-4			
Thomas and Breinl.						11 and 18 (2 rats).	4 animals inoculated with negative results.			S. C.	6-11	days 24-108†	One goat inoculated with negative results.		
Uhlenhuth, Hübener, and Woithe (Os tertag's strain).	I. P.	6	8	I. P.	4	5						Acut., 39-56 days. Chronic, 6 mos.		Dogs. days 48 and 103 (2 dogs). Horse. 153	
Browning.		1-2	5-7												
Pearce and Brown.	I. P.	24-36 hrs.	4-8	I. P.	36-48 hrs.	7-8	I. P.		5-7†	I. V.	10-18 (S.)	wks. 3-6			

* Some animals refractory to infection. † Some animals alive at 6 mos. ‡ Possibly much longer.

TABLE VI.
Tr. rhodesiense (Sleeping Sickness).

Investigators.	Mice.			Rats.			Guinea pigs.			Rabbits.			Other animals.		
	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.
Monfort. Mesnil and Riegenbach. Laveran.		<i>hrs.</i>	<i>days</i> 4 4-5		<i>days</i> 2-3	<i>days</i> 9* (7-12†) 8½* (6-18†)		<i>days</i> 8-10	<i>days</i> 41* (19-89†)					<i>days</i>	<i>days</i>
	I. P.	24	6½	S. C.											Dog (1). 15
	S. C.	48												4-5	Dogs (4). 9-12
Yorke.															

* Average. † Extremes.

TABLE VII.
Tr. dimorphon (Gambian Horse Disease).

Investigators.	Mice.			Rats.			Guinea pigs.			Rabbits.			Other animals.		
	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.
Laveran and Mesnil.		days 8	days 23* (10-42†)		days 3-12	days 24 and 30 (2 guinea pigs.)		days 9* (4-15†)	days 76 and 115 (2 rabbits).			days			days
Dutton and Todd. Monfort.	2-7	16-30 4	18* (7-42†)			13-60	I. P.			I. P.					Dogs. 10-19
Thomas and Breinl.	16-130						I. V.	4-7	26-35†	I. V.					Young dogs. 36

* Average. † Extremes. ‡ More chronic type, 78-157 days.

TABLE VIII.

Tr. congolense.

Investigators.	Mice.			Rats.			Guinea pigs.			Rabbits.			Other animals.		
	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.
Laveran.		8-30 days	105* (18-331††)			19* (15-29†§)		7-8 days	14* (9-24†)			27 and 70 days (2 rabbits).	S. C.	Dogs. 10-15 days	34* (21-52† days)

* Average. † Extremes. ‡ Usually ends fatally in mice. § Always fatal in rats. || Not always fatal in rabbits.

Acute Blood Infections of Mice and Rats.

The experimental infection of pathogenic trypanosomes in mice and rats is, generally speaking, an acute, progressive, and fatal disease characterized by the constant presence of parasites in the peripheral blood. With most strains, the parasites multiply continuously and quite regularly from the time they first appear in the circulation until death, when they seem to be more numerous than the red corpuscles. Signs or symptoms of the infection in these animals are rarely seen up to within a few hours of death. The incubation period, the interval of time between the inoculation and the first appearance of trypanosomes in the peripheral blood, varies in any instance with the species of organism, the size of the inoculating dose, the potency of the virus, and the route of inoculation. With the intraperitoneal and intravenous routes the incubation period is shorter than with the subcutaneous and, generally speaking, the more virulent the species of trypanosome, the shorter is the incubation period. We have used our strain of *Tr. brucei* for all routine work and a description of this infection will serve as a type of the acute blood infections of mice and rats.

Within 24 hours after intraperitoneal inoculation in mice of a small dose of infected blood (0.2 cc. of a + suspension), parasites are found in the circulating blood (+ grade). From the time of their appearance, their number constantly and regularly increases and in 48 hours, the infection is usually of a +++ grade. On the 3rd day (60 to 72 hours), it is ++++ and the parasites approach the number of the red blood corpuscles. With an infection of the virulence and severity of our strain of nagana, mice will die at the end of the 3rd or the beginning of the 4th day. The animals appear perfectly healthy up to the time of death. This may be ushered in by a short period of excitement with tremors, jerking of the limbs, and convulsions, which may be of extreme degree; or the mice may appear drowsy just prior to death and die without convulsions. Rats also, in our experience, may have convulsive attacks before death or may become drowsy during the last hours of life and die without other signs.

The infection produced by *Tr. brucei* in rats is quite similar to that in mice, with the exception that rats live longer than mice after the

number of parasites in the blood has reached a + + + + grade. The incubation period after intraperitoneal inoculation is slightly longer. The rapidity of development of the infection is quite comparable with that observed in mice and a + + + + grade is reached within 3 to 4 days. With such an infection, rats will live from 1 to 2 days, thus dying 4 to 6 days after inoculation.

The infection produced in mice and rats by other species of pathogenic trypanosomes which we have used is comparable with that of *Tr. brucei* as regards the general type of blood infection, although there are certain minor variations which should be considered. Infections with *Tr. equiperdum* and *Tr. evansi* are somewhat less acute than with *Tr. brucei*, mice living from 4 to 8 days and rats from 7 to 8 days. Occasionally the progressive course of a dourine (*Tr. equiperdum*) infection will be interrupted by a day or two in which decreasing numbers of parasites are found in the circulating blood. The incubation period for both *Tr. equiperdum* and *Tr. evansi* in mice is from 24 to 36 hours and in rats is usually slightly longer (approximately 36 hours), after intraperitoneal inoculation. Infections produced with our strains of *Tr. gambiense* are more chronic in character, mice dying in 5 to 8 days and rats in 5 to 11 days. It is uniformly fatal for both species of animals. The incubation period for mice is 24 to 48 hours and for rats usually 48 to 72 hours after intraperitoneal inoculation. With our strain of *Tr. equinum* also, a slower type of infection is produced, mice and rats usually dying in 5 to 8 days with an incubation period of 24 to 48 hours. Occasionally the infection may run a curious relapsing course. On the 4th or 5th day, the parasites in the peripheral blood will greatly diminish in number for 2 to 4 days. At the end of this time, they will recur with increasing rapidity and death will follow in 10 to 14 days after inoculation. We have noted this relapsing type of caderas infection more frequently in mice than in rats, but, on the other hand, we have seen many more caderas mice than rats. Occasionally also, we have seen spontaneous recovery in rats infected comparatively lightly with *Tr. equinum*, but this is not the rule with our strain of this organism. Laveran and Mesnil draw attention to signs and symptoms of approaching death in surra, caderas, and dourine mice which are not usually shown by nagana animals. The mice are very quiet and sit huddled up with

a rough and bristling coat. The animals are insensitive to external stimuli, the eyes are usually closed, and the corneas may become white and opaque just prior to death.

Chronic Tissue Infections of Guinea Pigs and Rabbits.

Experimental trypanosomiasis of rabbits, and to a certain extent of guinea pigs also, constitutes a type of infection in which tissue involvement is a conspicuous and predominating feature, and in this respect contrasts sharply with the blood infection of mice and rats. In neither guinea pigs nor rabbits is the presence of parasites in the peripheral blood a constant characteristic of the infection and in rabbits, in our opinion, it is of distinctly minor importance. The visible or external signs of tissue involvement of guinea pigs, it is true, may not be so striking or so constant as they are in the rabbit except in certain chronic infections of long duration. On the other hand, the infection is not of the acute type seen in mice and rats in which the parasitic invasion of the peripheral blood stream is the characteristic and constant feature. Consequently, guinea pigs would seem to be more appropriately classed with rabbits in the group of chronic or subacute tissue infections.

Trypanosomiasis in Guinea Pigs.—Guinea pigs inoculated intraperitoneally with our strain of *Tr. brucei* (0.5 cc. of a + suspension) will show a few parasites in the circulating blood within 3 to 7 days. The number of trypanosomes increases slowly for a few days reaching a ++ to +++ grade in about a week after their first appearance, when quite suddenly they disappear from the peripheral blood and one may examine a large number of films without finding a single trypanosome. This condition continues for an irregular period of time, usually 1 to 3 weeks during which, although one may find an occasional organism in blood films, the examinations are usually negative. The parasites invade the peripheral blood stream again as suddenly as they left it and with this second appearance increase rapidly and fairly uniformly in number reaching a +++ to a ++++ grade of infection in about a week. Death usually occurs at this time and is comparatively sudden. It is generally ushered in by local and general muscular tremors, twitching of the limbs, respiratory distress,

general convulsions, and stupor with some spasticity lasting from $\frac{1}{2}$ to several hours. The average length of life of guinea pigs infected with our strain of *Tr. brucei* is 4 weeks, although many have died in 2 to 3 weeks and others have lived 2 months. With a smaller inoculating dose than we usually use and a consequently less acute and more prolonged grade of infection, we have had some nagana guinea pigs which have shown clinical signs of the disease. In these animals, there has been slight loss of hair especially about the eyes and over the back, more or less edema of the external genitalia and anus, together with loss of weight or even emaciation. Such a chronic type of nagana infection, however, is unusual with our strain of *Tr. brucei*, which is quite virulent for guinea pigs. Guinea pigs receiving massive inoculating doses of *Tr. brucei* usually die within a week's time and do not show the characteristic cyclic appearance of parasites in the circulating blood. Such an overwhelming infection is comparable with the acute type seen in mice and rats, although in the case of guinea pigs, the uniformly progressive increase of trypanosomes in the blood may not occur.

Infections in guinea pigs with our strains of *Tr. equiperdum* and *Tr. evansi* are similar in general to *Tr. brucei* infections, although with both of these organisms, the course of the disease is of somewhat longer duration. The characteristic cyclic invasion of the peripheral blood stream by trypanosomes is seen in infections with both *Tr. equiperdum* and *Tr. evansi*, but with *Tr. equiperdum* especially there may be more than one period of remission in which no organisms are found in the blood. With our nagana strain, guinea pigs usually die at the height of the second cycle, or invasion of the peripheral blood, but in *Tr. equiperdum* infections, there is a tendency toward more than two cycles. The succeeding cycles in which one may find parasites are usually shorter than the earlier ones. The length of life of guinea pigs infected with *Tr. equiperdum* is quite irregular but averages from 5 to 7 weeks. Guinea pigs infected with *Tr. evansi* live on an average of 4 to 7 weeks but here again, there is considerable irregularity and definite statements are difficult to make. We have had no spontaneous recoveries with either strain in this species of animal. Chronic infections with *Tr. equiperdum* and with *Tr. evansi* are not rare and may be very prolonged, in which case clinical signs

such as the loss of hair, chronic lesions of the skin, and edema of the genitalia are not infrequently seen, an extreme grade of which is shown by the dourine guinea pig in Figs. 1 and 2. This guinea pig lived 106 days and showed conspicuous signs of the disease, consisting of extensive loss of hair with scaling and thickening of the skin, marked edema of the eyelids, external genitalia, and anus, and a profuse mucopurulent nasal discharge. The animal was extremely weak, thin, and emaciated, with muscular tremors, and blood films showed large numbers of trypanosomes.

Infections in guinea pigs with our strain of *Tr. gambiense* are extremely irregular both as regards the cyclic character of the infection and the length of life of the animal. As a rule, the condition is outspokenly chronic, guinea pigs surviving from 2 to 6 months and sometimes as long as 1 year. The periods of remission in which no parasites are found in the peripheral blood are usually considerably longer than those of guinea pigs infected with other pathogenic trypanosomes. The number of cycles is very irregular and even average estimates are difficult to make. Clinical signs and symptoms may be present in infected animals of long survival, but they are not so prominent or so conspicuous in our experience as in the chronic infections of other trypanosomes. Spontaneous recovery of lightly infected guinea pigs is not uncommon. One such guinea pig in our possession has lived over 4 years and shows no signs of the disease.

Infection of guinea pigs with our strain of *Tr. equinum* is also a chronic condition, similar to that of *Tr. gambiense*, although the average time of survival is somewhat shorter. Guinea pigs usually live from 1 to 2 months, although survivals of 3, 4, and 5 months are not at all uncommon. We have had no instance of spontaneous recovery of guinea pigs with our caderas strain. External tissue involvement is confined, in our experience, to edematous swelling of the scrotum and sheath. The cyclic invasion of the peripheral blood by trypanosomes is a characteristic feature, but as with *Tr. gambiense*, the infection is extremely irregular.

Experimental trypanosomiasis of guinea pigs, therefore, presents certain features which contrast sharply with the infection of mice and rats. There is a characteristic cyclic invasion of the peripheral blood by trypanosomes and in the periods of remission, no parasites are

found. Although the number of trypanosomes increases in the blood during these cycles of blood invasion, this increase is by no means so regular or so uniformly progressive as is usually the rule in the blood infections of mice and rats. While the length of survival of guinea pigs is extremely irregular, generally speaking it is a matter of weeks and months with the ultimate possibility of spontaneous recovery. In addition, in the more chronic infections, clinical signs may appear, all of which is in striking contrast to the infection in mice and rats with the same organisms. These features of the infection in guinea pigs are indicative of two conditions of chemotherapeutic significance: first, the interposing of animal resistance to the progress of the infection, and second, the establishment of true tissue lesions, neither of which, with rare exceptions, is in evidence in either the mouse or rat infection. One of these factors, that of animal resistance, reaches its highest point in the guinea pig; the other, that of tissue involvement and reaction, is most typically developed in the rabbit.

Trypanosomiasis in the Rabbit.—Trypanosomiasis in the rabbit as in the guinea pig is an infection of considerable duration but characterized chiefly by the clinical signs and manifestations of disease, while the finding of trypanosomes in the circulating blood plays a distinctly minor part. Thus, in an advanced condition of rabbit trypanosomiasis with pronounced clinical signs and symptoms, no trypanosomes or, at best, only a few may be found in blood films to indicate the existence of an infection. In the majority of the experiments, we have used a strain of *Tr. brucei* carried constantly in stock guinea pigs and the rabbits were inoculated intravenously with 1 cc. of a + blood suspension per kilo of body weight. In these infections the initial signs of the disease usually appeared in from 5 to 7 days and the length of survival of the untreated animals was from 1 to 3 months. More animals died within the first 5 weeks, however, than survived for a longer period. The infection is uniformly fatal in rabbits.

The earliest signs of nagana infection in rabbits appear about the head and external genitalia. Slight puffiness of the upper eyelids, swelling of the lips, reddening and slight thickening of the base of the ears, together with a similar appearance of the external genitalia, may all occur within 1 week after inoculation. Usually, however, only one or two parts of the body are at first involved but within a

few days, new areas are affected. The swelling of one or both upper eyelids with injection of the conjunctival vessels is a common initial sign and may be the only one for several days (Figs. 3 and 4). This condition increases rapidly and the lower lids may also become involved although usually less severely. In a few days, the eyes are not infrequently completely closed (Figs. 5 and 6). By this time, the margins of the lids are considerably reddened, the eyelashes are falling out, and there is a thick yellow exudate gluing the lids together.

Involvement of the base of one or both ears is likewise a characteristic early sign and is easily detected by holding the ear up against the light. Extending upward from the base of the ear, one can see a faint reddening of the tissues with dilatation and congestion of the blood vessels. A day or two later, the base of the ear is distinctly thickened and feels warm to the touch. The swelling rapidly increases both in extent and degree until the ear begins to droop (Figs. 7 and 8), and in a few days it is so swollen and heavy that the animal can no longer hold it erect (Figs. 9 and 10). The hair over the swollen area soon falls out and the skin becomes brawny, rough, and scaly. Crusts and scabs form and not infrequently actual ulceration of tissue occurs (Figs. 11, 13, and 14).

About the time that the eyes are partially closed and the ears beginning to droop, subcutaneous swelling of the face and upper lips appears (Figs. 11 and 12). In some rabbits, however, swelling of one upper lip was the first sign of the disease. At first, these swellings are rather puffy and edematous, but they soon become hard, indurated, and markedly reddened. In the beginning, they are small and focal, the entire lip itself not necessarily being involved but they rapidly increase in size, affecting the entire lip and not uncommonly both lips. A similar development may occur in the facial swellings which may become so marked that the contour from the base of the ear to the nose and lips is an extreme convex curve. The swellings of the face and lips, however, may remain more localized and when the early edema has disappeared, the deeper tissues are seen to be still involved. This is noticeably the case over the bridge of the nose in which swelling and induration of the periosteum with subsequent ulceration is not uncommon. There have also been a few instances of circumscribed swellings toward the end of the nose which are prone

to secondary infection. Accompanying the facial and lip involvement, there is a nasal discharge which usually begins in the 2nd or 3rd week of the infection. This discharge is at first mucoid in character and usually slight in amount but as the disease progresses, it may become very profuse with the formation of thick yellow crusts about the nostrils (Fig. 23).

Involvement of the external genitalia practically always occurs either as an early or late sign of the disease. In the majority of our rabbits, it occurred early and not infrequently was the first sign noted. In male rabbits, slight swelling and reddening of the prepuce or slight enlargement of one or both testicles are the initial signs (Fig. 15). The testicle feels hot, edematous, and somewhat elastic. In a day or two, it may become enlarged to two or three times its normal size (Fig. 16). The scrotum is extremely tense and glistening and the testicle itself becomes markedly resistant and hard. As the involvement advances, the actual swelling usually decreases somewhat but the testicle becomes more indurated and rubbery with a brawny, thickened scrotum (Figs. 17 and 18), which may finally lead to superficial excoriation or even ulceration (Fig. 17). The condition of the prepuce is similar to that of the scrotum and testicles. At first, there is considerable swelling, congestion, and induration (Fig. 19) while in advanced grades of infection, widespread ulceration may occur (Fig. 20). Occasionally, the penis becomes swollen and indurated with but little involvement of the prepuce (Fig. 21), or the prepucial condition may practically clear up to be followed by the enlargement of the penis. In female rabbits, the external genitalia are similarly affected (Fig. 22). The labia are markedly swollen and congested and the vaginal mucous membrane becomes involved in the same process. The anus practically always shows some swelling and induration, although usually this is not a particularly early sign of the infection, but follows the swelling of the external genitalia. Diarrhea is not an uncommon symptom and occasionally a white mucous discharge from the rectum occurs, suggesting the involvement of the intestinal mucosa in the infectious process.

A certain proportion of rabbits showing some or all of the signs of nagana infection described above usually die from 3 to 6 weeks after inoculation. In the last 2 weeks, they become emaciated and much

weakened and eat but little (Fig. 25). The picture of such advanced infections is very characteristic (Figs. 18, 23, and 24). When, however, the development of the infectious process is less rapid, with a consequently longer survival of the animal, a more chronic pathological condition occurs. These rabbits survive 2 to 3 months and show an exaggeration of the local signs and symptoms of the advanced and less chronic disease. The most striking signs in such animals are the loss of hair over large areas of skin and necrosis of both soft and bony tissues (Figs. 26, 27, and 28). Most of the face, for instance, as in Fig. 26, may be entirely denuded of hair. The skin is scaly, is greatly thickened, and has lost its elasticity. It is difficult to pick up. The testicles and prepuce may become extremely indurated and on palpation, one gets the impression of fibrosis of the testicular tissue. In the rabbit shown in Fig. 27, there is a conspicuous deep ulceration over the bridge of the nose. Not only have the soft parts in this area been completely destroyed but the cartilage and bone are beginning to show necrosis. In this particular animal, there was little secondary infection of the ulcerated area but this may occur to a marked degree as shown in Fig. 28. This rabbit was an extreme example of a chronic trypanosomal infection. There was an extensive ulceration of the soft tissue of the entire face, nose, and lips with the formation of thick adherent crusts. The mucopurulent discharge from the nose was especially profuse and foul smelling. In addition to the striking condition of the face, there was also marked involvement of both ears, both upper eyelids, and external genitalia together with great weakness and emaciation. Loss of hair in areas other than the face is a common sign in chronic infections. It is perhaps most frequent on the legs and may extend down to the feet and toes (Figs. 29 and 30). The denuded skin becomes considerably thickened and scaly and superficial ulceration may occur with a slight sticky yellow exudate as well as some bleeding and the subsequent formation of crusts. In a few instances, we have seen a patchy loss of hair on the backs of rabbits.

Rabbits infected with other strains of trypanosomes have shown much the same clinical picture as those infected with *Tr. brucei*. Rabbits infected with comparable doses of our strain of *Tr. gambiense* (1 cc. of a ++ or +++ suspension per kilo of body weight) survived an average of 8 weeks. The first signs of the infection were noted 7

to 15 days after inoculation. With this strain of *Tr. gambiense*, we have been struck with the great frequency and comparative severity of genital involvement. In one series of nineteen rabbits, this was the first sign noted in twelve and it persisted in all of them with relatively slight involvement of the face and head. However, it should be stated that our nagana rabbits greatly outnumber those infected with *Tr. gambiense* and the above observation may not be true for a larger series.

Rabbit infection produced by our strain of *Tr. equinum* is quite comparable with *Tr. brucei* as regards length of survival and clinical signs, but in this connection it should be noted that the inoculating doses of *Tr. equinum* are purposely larger than those of *Tr. brucei*. Our experiments with infections produced by *Tr. equiperdum* and *Tr. evansi* in rabbits are not extensive enough to justify any effort to analyze these infections in detail. As far as our experience has gone, the clinical course of the disease caused by these organisms in rabbits is similar to that of nagana.

These descriptions of trypanosomiasis in laboratory animals are, of course, but type descriptions of the infection as it usually occurs and it should be appreciated that the experimental disease is subject to very marked alteration in part due to "natural" or inherent causes but in part also due to causes subject to the control of the investigator.

Some Factors Influencing the Course of the Infection.

In addition to the animal species as a determining factor in the type of trypanosomal infection, there are certain other factors which may influence the character and termination of the infection of a given organism in any particular animal species. First, the dose of infecting organisms, other things being equal, may be so regulated as to insure within reasonable limits the general character of the infection together with the desired length of life of the inoculated animal. This is comparatively easily obtained in mouse and rat infections with the more virulent organisms and may be quite closely approximated with the less virulent species. With larger animals, in which as we have emphasized, a different type of infection obtains, such exactness is a more difficult task but by regulating the size of the inoculating

dose, one may produce a relatively rapid and acute infection or a more prolonged diseased condition or with the less virulent organisms, especially, a mild grade of infection which may end in spontaneous recovery. To meet the requirements of a more exact dosage, Kolmer (22) has devised a method of counting the trypanosomes in order to inoculate animals with known numbers of organisms. The mode or route of inoculation merely affects the period of incubation, the parasites appearing in the blood or the signs of the infection developing sooner after intraperitoneal or intravenous than after subcutaneous inoculation.

Secondly, the virulence of different species of trypanosomes must be taken into account. Generally speaking, *Tr. brucei* is naturally the most virulent species for the more common laboratory animals. *Tr. dimorphon*, *Tr. congolense*, *Tr. equiperdum*, and *Tr. equinum* are among the less virulent and between these extremes in varying degree are *Tr. evansi*, *Tr. gambiense*, and *Tr. rhodesiense*. In addition, the virulence of various strains of the same species must be considered. Although a freshly isolated strain is usually less virulent for laboratory animals than one that has been carried in stock animals for some time, this may not always be the case. A freshly isolated strain of *Tr. gambiense*, for instance, may be highly virulent for a large number of animals, while another freshly isolated strain may produce a chronic or subacute experimental infection in laboratory animals which may or may not end in spontaneous recovery. Moreover, different strains of the same species may show considerable variation in virulence for a given animal species. With certain strains of *Tr. equiperdum*, for instance, mice and rats are easily infected, while with others, the infecting power for these animals is absent or of a very low grade. Most strains of *Tr. brucei* are highly virulent for mice, killing them in 3 to 4 days, yet the Uganda strain of Bruce (23), kept in stock mice, killed regularly in 10 to 12 days.

The factor of acquired virulence of a particular strain must be kept in mind as well as its "natural" or inherent virulence. The virulence of any strain is subject to experimental influences as by its "direct" passage in the same animal species or by "crossed" passage in different species as well as by its rate of passage. Thomas and Breinl (17), speaking of *Tr. gambiense*, state that "if a strain be re-

peatedly and quickly run through animals of the same species, it will acquire a certain virulency for such a species, but this continues only so long as the strain is not run through other species of animals." However, according to Laveran (24), who gives the following résumé of his experiments, this augmentation of virulence by continued direct passage is not characteristic for all species of trypanosomes: "For *Tr. evansi* (surra of Mauritius and of Mbori) and for *Tr. gambiense*, virulence is increased following a series of passages in guinea pigs. For *Tr.* of Togoland (nagana), the virulence is diminished and for *Tr. congolense*, the virulence is not varied in spite of the great number of passages." It should be noted, however, that these experiments dealt with the guinea pig, an animal which in our experience may give the most varied results depending upon the uncertain factor of the infection cycle. According to other authors, the virulence of a strain by continued direct passage in the same animal species may be modified by this procedure for other animals as well. The modification may take the form of an increased or of an attenuated virulence. Martini (25) reports experiments with a nagana strain (Togoland) which after passage in mice and rats was increased in virulence not only for mice and rats but for dogs, while on the other hand, the viruses of passage through Equidæ, rabbits, and guinea pigs were very slightly virulent for rats, mice, and dogs. Laveran and Mesnil (26) cite the case also of a strain of *Tr. equiperdum* which at first killed mice regularly in 5 to 10 days. This strain was then kept in stock guinea pigs for 2 to 3 months and at the end of this time transferred back to mice. But it had become so much less virulent for mice that the animals had only the slightest grade of infection which ended in spontaneous recovery and the strain was lost. As a general rule, however, it seems to be the consensus of opinion that for most strains of trypanosomes, continued direct passage in any one species of animal increases the virulence for that particular animal species and that crossed passage, from one animal species to another, usually decreases the virulence. Further, the examples of altered virulence in trypanosome strains cited above are striking illustrations of the influence of animal species, as measured by the infection type, upon the organism in question. In addition, the rate of passage of a given animal species is usually an influencing factor, since the rapid trans-

fer from animal to animal at the height of the infection tends to increase the virulence of the strain.

The viability or infecting power of the virus is still another factor which measurably influences the character and termination of any particular infection in a given animal. The blood used for inoculation should be taken from a live or freshly killed stock animal, since pathogenic trypanosomes soon lose their infective power after the death of the host as has been shown by a number of observers. The stage of the infection of the stock animal should also be considered. In the continually progressive infection of mice and rats, the number of parasites in the blood constantly increases, but immediately before or at the time of death of the animal, a number of the parasites show degenerative changes and loss of motility. After diluting the blood for inoculation, it is apparent that under these conditions inoculating doses, which contain comparatively few parasites, would be extremely irregular as regards their infective power. Moreover, toward the terminal stages of an infection, there are present in the blood certain "immune substances," the nature and amount of which are as yet little understood. If in an endeavor to compensate for the comparatively few infecting organisms in the inoculating dose, one increases either the size or the concentration of the dose, the amount of immune substances is also increased at the same time, so that in any case the infecting power of such blood in proportion to the number of trypanosomes may be considerably diminished. On the other hand, if the blood is taken at an early stage of the infection, when there are comparatively few parasites, it is difficult to make an even suspension and an irregularity in the infection produced will result. If subinoculations are to be made from guinea pigs, the cyclic type of infection, characteristic of this animal species, should be borne in mind. When the infection is at its height, just prior to a blood crisis in which the parasites disappear from the circulating blood, degenerative forms and agglomerations of the organisms are frequent. Obviously, this stage as well as the periods of remissions are not favorable times for subinoculations into series of animals in which regularity of incubation together with uniformity of character and termination of infection is of importance.

Resistance of Specific Organisms to Therapeutic Agents.

Another set of conditions which the chemotherapist must consider is introduced by the factors of specific and strain resistance of trypanosomes to therapeutic agents as far as these facts are known. There have been numerous examples of such resistance of different species to a particular therapeutic agent reported in the literature. In his first experiments on the therapy of experimental trypanosomiasis, Ehrlich noted that trypan red was less active in nagana than in caderas mice. Atoxyl, which has a marked action on *Tr. gambiense* infections according to Mesnil, Nicolle, and Aubert, acts only feebly on *Tr. dimorphon* (Wenyon, Laveran) and has no action whatever on *Tr. congolense* (Laveran) (27). Monfort (28), in treating infected mice with arsenophenylglycine, found from the point of view of relapses that *Tr. gambiense* infections were the most easily cured, *Tr. rhodesiense* followed, *Tr. dimorphon* were only fairly easy, while *Tr. congolense* infections were highly refractory to the drug, sterilization being only of short duration. Laveran (29), who studied the treatment of *Tr. dimorphon* and *Tr. congolense* infections in guinea pigs with arsenic trisulfide, noted that this compound which had but little action against the first infection was very efficacious against the second. Mesnil and Brimont (30) state that surra and dourine infections were less resistant to tartar emetic than were nagana, caderas, *Tr. gambiense*, and *Tr. dimorphon* infections. Danysz (31) found that *Tr. rhodesiense* infection in mice was more resistant to 88² than *Tr. evansi*, and in order to obtain the same curative result, it was necessary to use twice as large a dose. More recently, Yakimoff and Wassilevsky (12) found, in working with dourine infections in mice, that the ratio of the curative to the tolerable dose of 102¹ (luargol) was 1:3, while Danysz reported the striking ratios of 1:80 in surra mice and 1:100 in mice infected with *Tr. gambiense*. In general, with our five strains, we have found that *Tr. equinum* is the least resistant and that *Tr. gambiense*, *Tr. brucei*, *Tr. evansi*, and *Tr. equiperdum* follow in the order named.

The differentiation between the virulence and the resistance of species of trypanosomes should be borne in mind since a marked resistance to therapeutic agents is not necessarily accompanied by a

high degree of virulence and *vice versa*. The classical example of *Tr. lewisi* may be cited as an organism of extremely low virulence but of high resistance to all known therapeutic agents. Furthermore, it should be emphasized that the resistance of the various strains of a species of trypanosome is not constant and that the widest differences in the resistance of different strains may be encountered.

Finally, as Laveran and Mesnil and Ehrlich have pointed out, one cannot conclude from the therapeutic results obtained against a particular infection in one species of animals what the results will be against the same infection in another animal species. In our experience, both *Tr. evansi* and *Tr. equiperdum* are more easily dealt with in rats than in mice, a condition the reverse of which usually obtains.¹

Principles of Adaptation and Utilization; Conclusions.

We have endeavored to show that the use of experimental trypanosomiasis in chemotherapeutic experiments should be based upon a clear conception of the experimental disease as it occurs in laboratory animals and that the use of one or another of the experimental infections is not to be undertaken in a haphazard fashion but that there are definite principles upon which the experiments may be based.

These considerations may appear obvious, but as one searches through reports dealing with experimental chemotherapy of trypanosomiasis, it is impossible to find any clear-cut application, or even recognition of these principles as a whole, to the problem of chemotherapy. Some have emphasized one point and some another, but on the whole, the problem of adaptation has been conceived with a very limited regard for the principles set forth. In the first place, one must know the behavior of the various species of trypanosomes in the animal body, the character of the infection produced, and the response which may be expected from these organisms to the particular

¹ In speaking of drug resistance of different species or strains of trypanosomes, the phenomenon of drug fastness should be called to mind. The differentiation between natural and acquired resistance or fastness to drug action is extremely difficult and since so much experimental work has been done with the various laboratory strains of trypanosomes, it is now almost impossible to say whether the resistance to drug action exhibited by any of these organisms is natural resistance or one which has resulted from laboratory handling.

class of therapeutic agents to be employed, and further than this, one must know even the peculiarities of the particular strain to be used. It is of equal importance also that one should be familiar with the various factors which may influence these reactions and the means to be employed to insure constancy of reaction under all circumstances. Finally, one should realize clearly the limitations to the usefulness of any particular form of the experimental infection or species of trypanosome as well as the limitations to deductions which may be drawn from all classes of experiments.

The animal factor divides experimental trypanosomiasis of laboratory animals into two main classes: the acute blood infections of mice and rats and the chronic tissue infections of guinea pigs and rabbits. From the point of view of the chemotherapy of experimental trypanosomiasis, these two types of infection present totally different problems. Treatment of the acute infections of mice and rats is essentially one of speed, since the duration of the disease is but a matter of days and resolves itself into the treatment of a condition analogous to a bacteremia in which the multiplying parasites are predominately found in the circulating blood. A uniform and constant grade of infection is usually easily regulated in mice and rats and since relapses generally occur within 2 weeks after treatment, the more remote therapeutic effects are not delayed. On the other hand, certain factors of importance which are not included in the infection of mice and rats are supplied by trypanosomiasis of guinea pigs and rabbits. In the natural disease of human beings and of animals, tissue involvement is a conspicuous feature and the question of tissue penetrability must be taken into account. In experimental trypanosomiasis of the rabbit and of the guinea pig to a somewhat less extent, tissue involvement with the consequent clinical signs and symptoms predominates and the use of these animals directly supplies the necessary experimental conditions which are wanting in the infections of mice and rats. Not only have the parasites to be killed by the therapeutic agent in treating rabbit trypanosomiasis, but the involved tissue, the site of pathological changes produced by the invading trypanosomes, must be penetrated by the trypanocidal substance, and furthermore, the lesions themselves must be healed. In addition, the factor of duration of the experimental infection must be considered, especially from the

practical outcome of the chemotherapeutic problem, since trypanosomiasis of human beings and of most animals is essentially a chronic rather than an acute disease, lasting weeks, months, and even years. Emphasis should also be laid on the individuality and irregularity of rabbit and guinea pig trypanosomiasis which adds considerably to the value of these animals in any extensive therapeutic experiments. The treatment of infected rabbits especially, resolves itself into the treatment of individuals and such a plan of procedure is directly comparable with that pursued in dealing with the naturally acquired disease. As Strong and Teague (32) have pointed out in their paper on the treatment of surra in the Philippine Islands, the methods of treatment that have given satisfactory results in mice and rats have failed to cure larger animals. In order, therefore, to reproduce, with the usual laboratory facilities, experimental conditions analogous to the natural infections of man and animals, we have utilized the rabbit as the animal that most nearly fulfills the desired requirements. From this point of view, the results of treatment of chronic tissue infections are indicative of curative power or therapeutic potency as contrasted with trypanocidal action.

The selection of the species of trypanosomes should be based on a knowledge of the type and course of the infection produced in the various animal species and on its general resistance to therapeutic agents. The virulence of any particular strain is dependent largely on its inherent natural qualities but may be measurably influenced by certain extraneous factors such as the length of time of isolation and the method of propagation, which are subject to the control of the investigator.

Treatment of experimental trypanosomiasis of mice and rats is largely a matter of speed of action. Since these animals are easily procurable and the results of treatment are quickly seen, the chemotherapist has in this type of infection a valuable experimental test for the quick determination of the therapeutic activity of a compound. For all preliminary routine work, especially if a large number of compounds are to be tested, the utilization of mouse and rat infections gives definite and valuable information in a relatively short space of time. On the other hand, since experimental trypanosomiasis of guinea pigs and rabbits is predominately a chronic tissue

infection more nearly analogous to the naturally acquired forms of the disease, the factor of ultimate curative power or potency of a compound, rather than its immediate trypanocidal action, is emphasized in the treatment of such infections. From the point of view of the chemotherapist, the two types of infections supplement each other and the proper utilization and adaptation of each type constitute a logical basis for procedure in the chemotherapy of experimental trypanosomiasis.

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EXPLANATION OF PLATES.

PLATE 4.

FIG. 1. *Tr. equiperdum*. Chronic infection. There is loss of hair about the eye and over the nose and right lip. The skin in these areas is thickened and scaly.

FIG. 2. *Tr. equiperdum*. Chronic infection. There is loss of hair over a large area of the back, haunches, and legs with marked thickening and scaling of the skin. The anus, testicles, and prepuce are greatly enlarged and indurated.

PLATE 5.

FIG. 3. *Tr. brucei*. Very early infection. The upper eyelid is slightly swollen and puffy.

FIG. 4. *Tr. brucei*. Early infection. Both upper eyelids are very swollen and the eyes are partially closed. The base of the right ear is similarly involved and the ear is beginning to droop.

PLATE 6.

FIG. 5. *Tr. equinum*. Moderately advanced infection. The eye is partially closed, the face is swollen, crusts are beginning to form over the nose, and the ears are partly drooping.

FIG. 6. *Tr. brucei*. Moderately advanced infection. The eye is completely closed and the face between the eyes and over the bridge of the nose is swollen.

PLATE 7.

FIG. 7. *Tr. brucei*. Early infection. The left ear is swollen and thickened about the base and is hanging down.

FIG. 8. *Tr. brucei*. Moderately advanced infection. The condition of the left ear has increased and the right ear has become involved. Both upper eyelids are swollen and the left eye is partially closed. The face and lips are considerably swollen.

PLATE 8.

FIG. 9. *Tr. brucei*. Early infection. The left ear is slightly swollen and is beginning to droop.

FIG. 10. *Tr. brucei*. Moderately advanced infection. The right ear droops, the right eye is almost closed, there is marked puffiness of the upper eyelid, and the face is swollen.

PLATE 9.

FIG. 11. *Tr. brucei*. Moderately advanced infection. The left lip is swollen and enlarged. The left ear shows a loss of hair with scab formation of the skin along its outer margin.

FIG. 12. *Tr. brucei*. Early infection. The entire face is very much swollen, the lips slightly so. Eyelids and ears are not yet involved.

PLATE 10.

FIG. 13. *Tr. brucei*. Advanced infection. There is loss of hair together with an extensive scab formation along the outer margin of the left ear. Both ears hang down. (The left was held up in order that the picture might be taken.) The left eye is almost closed and there is marked swelling of the face.

FIG. 14. *Tr. brucei*. Moderately advanced infection. There is an extensive scab formation with loss of hair about the base of the left ear extending up along the outer surface and margin. The face over the bridge of the nose is somewhat swollen.

PLATE 11.

FIG. 15. *Tr. brucei*. Early infection. Both testicles are swollen, the left especially so.

FIG. 16. *Tr. gambiense*. Early infection. The right testicle is enormously swollen, the left slightly so. The scrotum is very tense.

FIG. 17. *Tr. gambiense*. The same rabbit as in Fig. 16, 4 days later. The right testicle is decreased somewhat in size and the left is now practically the same size. The scrotum has become indurated and brawny and on the right side, there is a superficial ulcer.

FIG. 18. *Tr. brucei*. Advanced infection. Both testicles are larger than normal and are exceedingly hard. The scrotum has become thickened and indurated. The prepuce is slightly swollen and is indurated.

PLATE 12.

FIG. 19. *Tr. brucei*. Early infection. Moderate swelling and induration of the prepuce.

FIG. 20. *Tr. brucei*. Advanced infection. Extreme swelling, induration, and ulceration of the prepuce. Moderate involvement of the testicles.

FIG. 21. *Tr. brucei*. Advanced infection. Swelling and induration of the penis and right testicle. The left testicle is slightly affected. Prepuce not involved.

FIG. 22. *Tr. brucei*. Moderately advanced infection. Swelling and induration of the vulva and anus.

PLATE 13.

FIG. 23. *Tr. brucei*. Advanced infection. Marked lesions of eyes, ears, face, and nose with thick crusts about nose and lips. There is a profuse purulent discharge.

FIG. 24. *Tr. brucei*. Advanced infection. The face is swollen and indurated and the eyelids are glued together with a purulent exudate. The base of the left ear is affected.

PLATE 14.

FIG. 25. *Tr. brucei*. Advanced infection. Marked general emaciation with few local signs. The eyelids and face are slightly swollen.

PLATE 15.

FIG. 26. *Tr. brucei*. Chronic infection. Loss of hair over face, nose, and about the eyes. The underlying skin is indurated and has lost its normal elasticity. Scab formation about the eyes.

FIG. 27. *Tr. brucei*. Chronic infection. Necrosis of the soft and hard parts over the bridge of the nose. Moderate involvement of the eyelids and base of ears.

FIG. 28. *Tr. brucei*. Long standing, chronic infection. Extreme necrosis of face, nose, and lips with secondary infection. Marked involvement of eyelids and ears.

PLATE 16.

FIG. 29. *Tr. brucei*. Chronic infection. Loss of hair with induration and scaling of the skin along the hind leg.

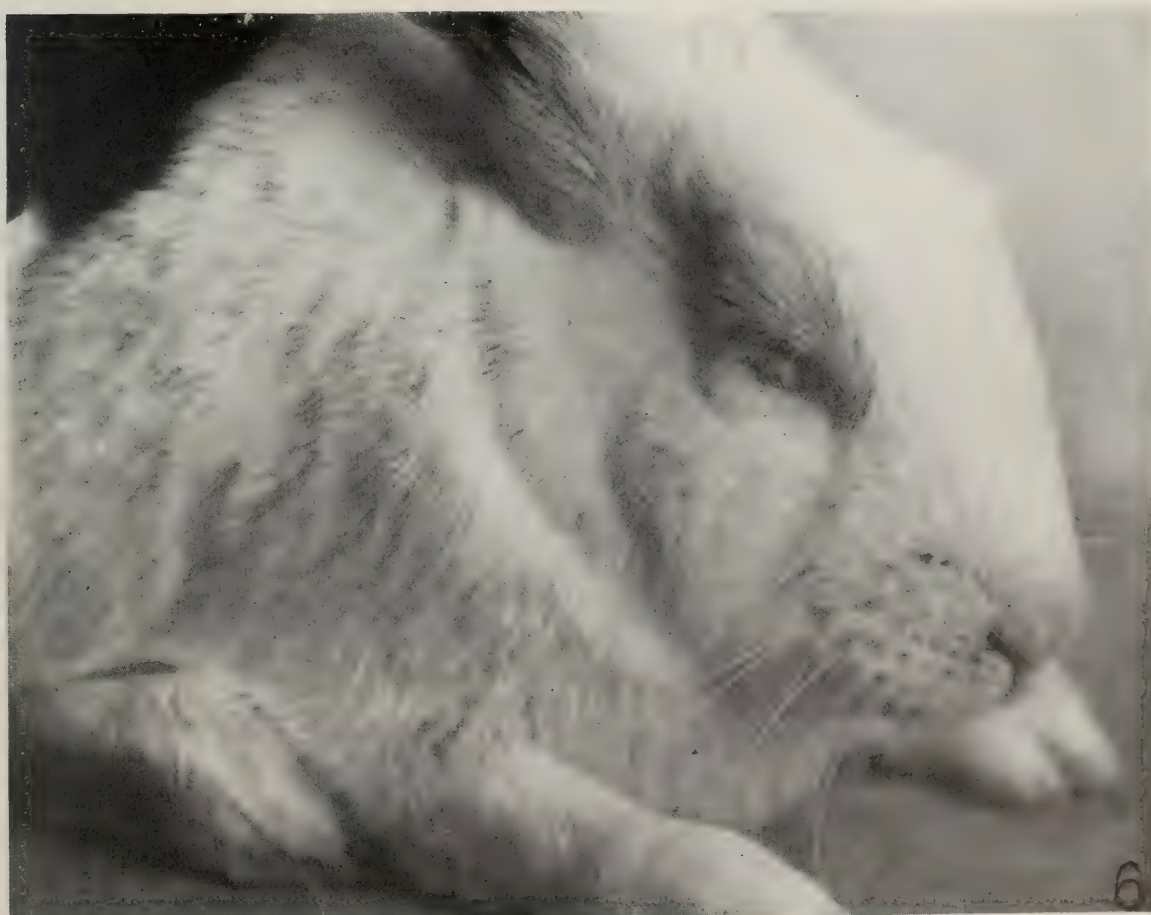
FIG. 30. *Tr. brucei*. Chronic infection. Loss of hair, induration of the skin, and superficial ulceration with scab formation of the leg.



(Pearce and Brown: Experimental trypanosomiasis.)



(Pearce and Brown: Experimental trypanosomiasis.)



(Pearce and Brown: Experimental trypanosomiasis.)



(Pearce and Brown: Experimental trypanosomiasis.)



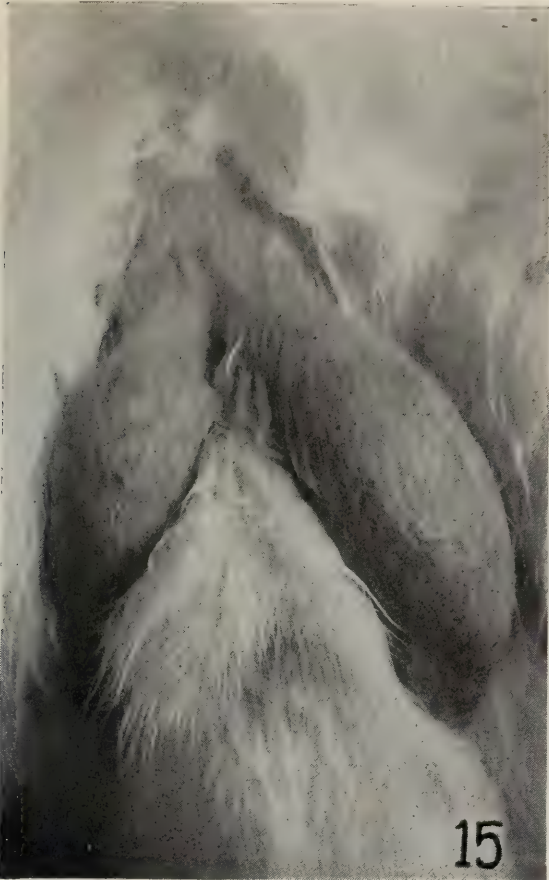
(Pearce and Brown: Experimental trypanosomiasis.)



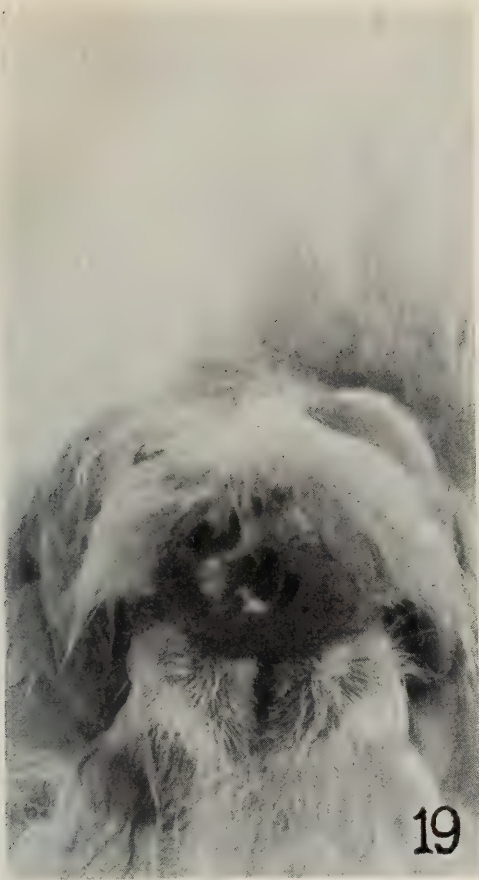
(Pearce and Brown: Experimental trypanosomiasis.)



(Pearce and Brown: Experimental trypanosomiasis)



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(Pearce and Brown: Experimental trypanosomiasis.)





(Pearce and Brown: Experimental trypanosomiasis.)

THE EFFECT OF PAINTING THE PANCREAS WITH ADRENALIN UPON HYPERGLYCEMIA AND GLYCOSURIA.

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INTRODUCTION.

About a year after the discovery by Blum¹ that subcutaneous injections of adrenal extracts produce glycosuria, Herter and his coworkers² published several communications on the sugar-producing effect of adrenal substance when administered intraperitoneally. They employed adrenalin chloride (Takamine, 1:1,000). According to Herter, intraperitoneal injections of adrenalin give better results than subcutaneous or intravenous injections. Herter's communications contain further the following striking statements: that painting of the pancreas with adrenalin brings on glycosuria which amounts often to 10 per cent and more, and that such a remarkable production of glycosuria may be brought about even by painting only one-fifth of the pancreas with adrenalin. He further states that "the pronounced nature of the glycosuria following intraperitoneal injections appears to be mainly attributable to the readiness with which the adrenalin comes into contact with the pancreas." The glycosuric action of painting the pancreas is brought into connection with the fact that glycosuria invariably makes its appearance after complete depancreatization, and the theory is advanced that the painting of the pancreas with adrenalin acts upon the cells of this organ in the manner of the action of hydrocyanic acid; namely, by the prevention of oxygenation of the glandular cells. In the discussion following this communication at the meeting of the Association of American Physicians, Herter stated that he was unable to offer a satisfactory reply to the question of one of us; namely, why the prevention of oxygenation of the cells of one-fifth of the pancreas should produce glycosuria, while the removal of even more than one-half of that gland produces no glycosuria. To the remarks of Cushing that the operative procedure,

¹ Blum, F., *Deutsch. Arch. klin. Med.*, 1901, lxxi, 146.

² Herter, C. A., and Richards, A. N., *Med. News*, 1902, lxxx, 201. Herter, C. A., *ibid.*, 1902, lxxx, 865. Herter, C. A., and Wakeman, A. J., *Tr. Assn. Am. Phys.*, 1902, xvii, 570; *Virchows Arch. path. Anat.*, 1902, clxix, 479. Vosburgh, C. H., and Richards, A. N., *Am. J. Physiol.*, 1903, ix, 35.

etc., might have been responsible for the glycosuria, Herter said that he "used only ether, and after you have used this for hours on dogs and examined the urine you get only a trace of sugar or none whatever." He admits that in a few instances the amount of glycosuria is inconsiderable and in exceptional instances glycosuria may be entirely absent. However, Herter's papers do not contain figures recording the number of successful experiments after painting the pancreas or after intraperitoneal injections, or indicating the degrees of the results.

In Herter and Wakeman's communications² there is only an unimportant reference to the hyperglycemic effect of painting. But later Vosburgh and Richards² report eight experiments in which the hyperglycemia was studied, five after painting the pancreas and three after intraperitoneal injections of adrenalin chloride. In these instances the increase in hyperglycemia was, indeed, considerable; but no statement is made as to the glycosuric effects. Vosburgh and Richards acknowledge the influence of ether upon the sugar content of the blood and the necessity of having controls for the experiments; they consider as a control the first analysis for blood sugar which has been made after anesthesia has been established and before adrenalin is used. In two of these controls the glycemia was high (0.239 and 0.258 per cent).

These statements are striking. Although more than 14 years have passed the experiments seem not to have been confirmed by other workers. We report below the results which were obtained in the repetition of some of the experiments.

EXPERIMENTAL.

Method.—The experiments were made on dogs. They were anesthetized with ether, some by the intratracheal insufflation method, some received ether by cone or towel, and in others ether was insufflated through a cone. In all cases urine and blood were analyzed for sugar content, the blood was analyzed once before painting the pancreas with adrenalin (Parke, Davis and Company, 1:1,000), and several times at intervals after painting. Blood was obtained from a cannula in an artery (carotid or femoral) or from the external jugular vein by means of a syringe, and the urine from a catheter kept permanently in the bladder. Probably because of the etherization, the amount of urine obtained was usually small. The experiment was observed for several hours; that is, at least until the glycemia was distinctly on its descending course. The urine was examined at intervals after painting and in some instances the urine excreted during the following 18 or 20 hours was collected and examined. Etheriza-

tion was continued either throughout the entire experiment or until the painting and with it the operative handling of the animal was finished. The quantitative analysis for sugar was made by Myers and Bailey's modification of the Lewis-Benedict method.³

After laparotomy the pancreas was exposed to as full a view as possible. At least one-third of the organ was painted. In some experiments the entire quantity of the adrenalin was painted on at once; in others about the same quantity was used but divided for painting several times. The results of the two methods are presented in Tables I and II.

Since the adrenalin will reach the pancreatic cells more thoroughly if applied after the thin membrane and the external covering of connective tissue have been removed, this was done in most of the experiments.

The results will be found in compact but full detail in the tables. Table I presents those obtained in seven experiments in which the pancreas was painted only once with fairly large quantities.

As regards the blood sugar we are concerned essentially with the determinations made during etherization just before exposure of the pancreas and the highest percentage of the sugar found at any time after painting it. The difference between these two determinations indicates the effect of painting the pancreas with adrenalin. Taking the highest increase during an experiment as characteristic of the effect of the painting, the rise in the blood sugar content in the experiments of Table I is as follows: 0.09, 0.07, 0.05, 0.06, 0.00, 0.08, and 0.06 per cent. It varies between 0.09 and 0.00 per cent. The urine never contained reducing substances before painting the pancreas with adrenalin. The highest percentages of glycosuria after painting in the experiments of Table I are as follows: 1.27, 5.00, 0.00, 0.93, 0.68, 0.94, and trace. As can be seen from the data in the table, the percentages of sugar in the urine following the painting were irregular, the highest being 5 per cent and the lowest 0.00 per cent. Evidence of definite relation between glycosuria, hyperglycemia, and the amount of adrenalin used in painting the pancreas is lacking.

Table II summarizes five experiments in which the pancreas was painted several times. The entire quantity of adrenalin used in

³ Myers, V. C., and Bailey, C. V., *J. Biol. Chem.*, 1916, xxiv, 147.

TABLE I.

Application of Adrenalin to the Pancreas Once.

Experiment No.	Weight Sex	Part and proportion of entire pancreas painted.	Amount of adrenalin (1:1,000 solution).	Blood sugar.				Urine.				Albuminuria.	Anesthesia.	Temperature of dog	Remarks.
				Before painting.	After painting.	Time.	Percent- age.	Length of period.	Amount.	Sugar.					
				Before ether.	During ether.	hrs. min.	per cent.	hrs. min.	cc.	per cent					
1	16.4 Male.	About one-third (posterior end and body). About one- half of both sides stripped of connective tissue cover- ing.	2.75	0.11	0.12	14	0.15	}	14.5	1.27	None be- fore. +	Ether (intra- tracheal in- sufflation) until 53 min. before last blood sample.	38.2 39.1 38.4		
						44	0.13								
						1 44	0.21								
						→ 2 45	0.15								
2	9.25 Female.	Entire pancreas. Not stripped of connective tissue cover- ing.	3.0	0.15	0.29	5	0.33	}	10.5	4.65	None be- fore. +	Ether (intra- tracheal in- sufflation) throughout.	37.5 36.6 35.9	Died during night. Duo- denum, cecum, and colon in- tensely hemor- rhagic.	
						15	0.31								
						31	0.33								
						1 1	0.33								
						2	0.34	}	15	5.00	+	Ether (intra- tracheal in- sufflation) throughout.	36.8 35.9 36.6	Died during night. Pneu- monia.	
3	0.36	2 1													
3	17.5 Male.	Entire pancreas (posterior end on only one side). Stripped only in one small area.	3.1	0.12	0.16	5	0.21	}	3.2	0.00	+ before. ++	Ether (intra- tracheal in- sufflation) throughout.	36.8 35.9 36.6		
						16	0.18								
						33	0.18								
						1 1	0.18								
						2 1	0.20	}	2.4	0.00	+				
3 2	0.21	2													

4	12.25	About two-thirds (posterior end and middle). Stripped only in two small areas.	2.4	0.12	0.23	6 16 32 1 2 3 24	0.27 0.29 0.28 0.28 0.27 0.13	1 2 21	0.0	None before.	Ether until 2 hrs. after painting (intracheal insufflation).	37.2 35.0 36.7 37.9 39.1	
5	14.5	About one-third (posterior end and middle). Partly stripped.	2.0	0.13	0.21	9 2 4 22	0.21 0.14 0.12 0.12	16 2 2 20	1.25 15.5 22 >188	None before.	Ether by cone for 44 min.	38.5 before. 38.3 38.9 38.5 38.8	
6	7.25	About two-thirds (anterior end and middle). Not stripped.	2.0	0.12	0.14	10 2 4	0.22 0.20 0.11	19 2 1 Post mortem.	0.0 0.94 0.0 0.0	+ before.	Ether blown through cone for about 50 min.	38.3	Died during night. Left lung somewhat congested.
7	9.6	One-third by weight (middle). Not stripped.	1.0	0.13	0.16	12 2 22	0.22 0.14 0.14	20 2 19	4 12 >38	+ before.	Ether blown through cone for about 39 min.	39.1 39.3	Vomited while under ether and again afterwards.

In the tables the arrow indicates the time when ether administration was discontinued.

TABLE II.

Application of Adrenalin to the Pancreas Several Times.

Experiment No.	Weight. Sex.	Part and proportion of entire pancreas painted.	Amount of adrenalin (1:1,000 solution).	Blood sugar.				Urine.			Albuminuria.	Anesthesia.	Temperature of dog. °C.	Remarks.
				Before painting.		After painting.		Length of period.	Amount.	Sugar.				
				Before ether.	During ether.	Time.	Per- centage.							
	kg.		cc.	per cent	per cent	hrs. min.	per cent	hrs. min.	cc.	per cent				
8	11.35 Male.	One-half (posterior end and part of middle). Ex- treme tip stripped.	2.0	0.14	0.28	→ 10	0.30	11	4	4.44	None be- fore.	Ether by towel for 41 min.	39.0 38.5	Vomited while coming out of ether. Pan- creas painted twice on one side and three times on the other side.
						2 25	0.21	1 54	13	6.95	Tr.			
						5 20	0.13	2 54	20	3.55	"			
						23	0.13	17	Tr.(?).	"				
9	11.05 Female.	One-fourth (poste- rior free end only). One side stripped.	1.5	0.15	0.24	→ 10	0.29	15	13.5	4.36	Bloody before.	Ether blown through cone for 57 min.	38.8 37.7 38.8 39.5 40.1	Each side of pan- creas painted twice.
						2 53	0.14	2 35	{ 8	2.70	Bloody.			
						4 34	0.13	1 31	{ 20.5	3.69	Tr.			
						22 36	0.12	18	26	Tr. 0	+			

10	10	One-third (posterior free end). Stripped to very small extent.	1.5	0.12	0.14	→ 2 10 4 33 24 32	0.17 0.12 0.13	15 2 13 3 4 18 50	2 15 25 240	0 Tr. 0 0	Bloody before. Bloody. + + +	Ether blown through cone for 34 min.	39.7 40.3 40.3 39.5	Each side of pancreas painted three times.
11	11.1	One-third of length (posterior free end). One side stripped.	1.5	0.13	0.24	→ 4 11 23 20	0.33 0.12 0.12	15 3 46 19 30	2.6 23 ?	3.08 1.81 0	Tr. before. ++ +	Ether blown through cone for 49 min.	38.8 38.2 38.0	Each side of pancreas painted three or four times.
12	11.1	One-sixth of length or one-fourth of weight (part of middle and very little of posterior free end). Both sides stripped.	1.5	0.12	0.18 12 min. later 0.20.	→ 2 10 4 40 22 35	0.24 0.13 0.11 0.14	16 2 20 1 55 18	3.6 20 25 ?	3.57 3.07 0.00 0.00	Tr. before. ++ ++ ++ ++	Ether blown through cone for 50 min.	39.0 38.0 39.0 39.2 38.5	Each side of pancreas painted four times.

the several paintings in each of the five experiments in Table II was generally smaller than those used in the experiments of a single painting in Table I. The rise of the blood sugar in the experiments of Table II is as follows: 0.02, 0.05, 0.03, 0.09, and 0.04 per cent. The highest percentages of sugar in the urine in these experiments were: 6.95, 4.36, trace, 3.08, and 3.57. The increase of the blood sugar is about the same in both tables. The glycosuria generally ran higher in the second series of experiments.

A consideration of both series shows that our experiments bear out in a general manner the statement made by Herter, that in the majority of the cases (nine out of twelve of our animals) painting the pancreas with adrenalin is followed by glycosuria, and further, the amount of the glycosuria varies in different experiments. However, our results do not support unqualifiedly the results presented by Herter and the views expressed by him. As regards glycosuria produced by adrenalin, Herter states that subcutaneous administration produces the least amount, intraperitoneal the next highest amount, and painting the pancreas the highest amount. The impression conveyed is that painting the pancreas will, as a rule, produce high glycosuria, 10 per cent and higher. Thus in one experiment 14 per cent was found, while the slighter glycosuria produced was exceptional and rarely the conditions failed entirely. Now our experiments give rather the opposite impression. Thus in one instance only did the glycosuria approach 7 per cent, while in another it was about 5 per cent. On the other hand, of the twelve experiments, three gave no glycosuria or a mere trace. In seven experiments the glycosuria was small or at least not high. The average of the twelve experiments was 2.23 per cent. In other words, we found the glycosuria following painting of the pancreas with adrenalin a less striking phenomenon and we are not convinced that it differs from the similar condition which follows the subcutaneous or intraperitoneal injection of the drug. Two more points must be considered. First, we employed larger amounts of adrenalin than apparently did Herter and his coworkers. Second, in most experiments we removed the connective tissue and the thin membrane from the part of the pancreas which was to be painted with adrenalin, a device which according to Herter⁴ is "apt to be most effective."

⁴Herter, C. A., and Wakeman, A. J., *Tr. Assn. Am. Phys.*, 1902, xvii, 577.

The experiments we have made would seem not to support the chief contention of Herter that the "pronounced nature of the glycosuria following intraperitoneal injections appears to be mainly attributable to the adrenalin which comes into contact with the pancreas."

Although Vosburgh and Richards² adopt the view of Herter that the glycosuria produced by injection of adrenalin into the peritoneal cavity is of pancreatic origin, they do not mention the point in the conclusion. As already stated, these authors studied only the effects of adrenalin upon glycemia. They made only three experiments, using large, toxic doses of the adrenalin solutions intraperitoneally; one animal died in 24 hours. We wish now to compare the hyperglycemia obtained in our experiments by painting the pancreas with the results obtained by Vosburgh and Richards. Their Table I contains five such experiments of which the main facts are reproduced in our Table III.

The blood sugar rises in their experiments are: 0.102, 0.118, 0.280, 0.240, and 0.052 per cent. Except in one experiment (No. 7) the rise in the blood sugar is therefore considerably higher than that obtained in our twelve experiments. The average increase in our experiments is 0.054 per cent; the average increase in those of Vosburgh and Richards is 0.158 per cent; thus the average of our experiments is about one-third that of Vosburgh and Richards. The doses of adrenalin employed in four of their experiments were larger than those in ours. Analysis shows, however, that the quantity of adrenalin employed by them did not exert an unmistakable effect upon the blood sugar. Thus in Experiment 4, in which 4 cc. of adrenalin were used, the increase amounted only to 0.118 per cent, while in Experiment 6, in which only 2 cc. were used, the rise was 0.240 per cent. Other points of difference between the methods employed by Vosburgh and Richards and by ourselves exist. The amount of blood withdrawn for each analysis is one of these points. Vosburgh and Richards undoubtedly have withdrawn much larger quantities of blood than we have withdrawn in our experiments. Possibly this is a contributing factor in their finding higher blood sugar than we found.⁵ The other possibility of a deeper anesthesia and some de-

⁵ For example, see Rinderspacher, K., *Biochem. Z.*, 1910, xxvii, 67-72.

TABLE III.

Blood Sugar Content in Five Experiments on Painting the Pancreas with Adrenalin from Table I of Vosburgh and Richards (Abbreviated).

Experi- ment No.	Time when blood was withdrawn.		Sugar.	Remarks.
			per cent	
3	3.00 p.m.	Normal.....	0.112	Etherization continued throughout experiment; 3 cc. of adrenalin chloride solution (1:1,000) applied to pancreas with brush at 3.08 p.m.
	3.15 "	7 min. after painting..	0.182	
	3.23 "	15 " " "	0.178	
	3.38 "	30 " " "	0.188	
	4.08 "	1 hr. " "	0.204	
	5.06 "	2 hrs. " "	0.214	
	6.00 "	3 " " "	0.165	
4	7.55 p.m.	Normal.....	0.173	Etherized from 7.45 p.m. till end of experiment; 4 cc. of adrenalin chloride solution (1:1,000) painted on surface of pancreas at 8.12 p.m.
	8.17 "	5 min. after painting..	0.277	
	8.27 "	15 " " "	0.237	
	8.42 "	30 " " "	0.291	
	9.12 "	1 hr. " "	0.256	
	10.12 "	2 hrs. " "	0.256	
5	10.45 a.m.	Normal.....	0.239	Ether given throughout experiment; 3 cc. of adrenalin chloride (1:1,000) applied at 10.55 a.m.
	11.00 "	5 min. after painting..	0.291	
	11.10 "	15 " " "	0.354	
	11.25 "	30 " " "	0.388	
	11.54 "	1 hr. " "	0.433	
	12.54 p.m.	2 hrs. " "	0.477	
	1.54 "	3 " " "	0.519	
	2.55 "	4 " " "	0.465	
6	4.52 p.m.	Normal.....	0.131	Ether given throughout experiment; 2 cc. of adrenalin chloride solution (1:1,000) applied to pancreas at 5 p.m.
	5.05 "	5 min. after painting..	0.205	
	5.15 "	15 " " "	0.217	
	5.30 "	30 " " "	0.264	
	6.00 "	1 hr. " "	0.315	
	7.00 "	2 hrs. " "	0.371	
7	3.34 p.m.	Normal.....	0.154	Ether given throughout experiment; 3 cc. of adrenalin solution applied to pancreas at 3.43 p.m.
	3.49 "	6 min. after painting..	0.192	
	4.01 "	18 " " "	0.173	
	4.22 "	39 " " "	0.191	
	4.58 "	1 hr., 15 min. after painting.....	0.206	
	5.43 p.m.	2 hrs. after painting...	0.143	
	6.37 "	3 " " "	0.169	

gree of asphyxia (factors which tend to increase the amount of blood sugar) is eliminated by the statement of Vosburgh and Richards that "care was taken to keep the anesthesia as light and as constant as possible." The differences in the method of analysis employed by Vosburgh and Richards (precipitation by phosphotungstic acid and determination of sugar by the Allihn method), and by ourselves (Lewis-Benedict method) cannot be responsible for the variations in our results.

However that may be, we are justified in pointing out the fact that the production of hyperglycemia by painting the pancreas with adrenalin in our experiments was not of unusual degree. There is reason to doubt that the glycosuria and the increase in the hyperglycemia thus produced are greater than would have been produced by the application of adrenalin to any other part of the peritoneal cavity. We have not established by direct experiments the degree of hyperglycemia and glycosuria which is produced by painting some other surface of the peritoneal cavity which would have entitled us to a direct comparison between painting the two surfaces.

On the other hand, we have ascertained definitely that the hyperglycemia and glycosuria produced by intraperitoneal injection of adrenalin is not of pancreatic origin. We have isolated in two series of experiments a part of the pancreas in such a way (1) that the adrenalin applied to the pancreas could not find its way to other parts of the peritoneal cavity at all or to any extent, and (2) that the adrenalin injected into the peritoneal cavity could not reach the isolated part of the pancreas. Table IV records the experiments in which the isolated pancreas was painted with adrenalin.

In the experiments in which the adrenalin was painted on the pancreas and was prevented from coming in contact with any other part of the peritoneum the rise of blood sugar was: 0.02, 0.04, 0.02, 0.06, 0.02, 0.01, 0.04, and 0.07 per cent, giving an average of 0.035 per cent, which is about two-thirds that obtained when the adrenalin was applied to a pancreas remaining in contact with the rest of the peritoneum. The glycosurias were: 0, 1.08, 0, 0, 2.66, 0.57, 0.7, and 0 per cent, with an average of 0.63, which is less than one-third the average of the glycosurias obtained when the painted pancreas remained unisolated from the peritoneum. These figures indicate that the increases

TABLE IV.
Pancreas Isolated; Adrenalin Applied to Pancreas.

Experiment No.	Weight. Sex.	Part and proportion of entire pancreas painted.	Blood sugar.						Urine.			Albuminuria.	Anesthesia.	Temperature of dog. °C.	Remarks.	
			Before painting.		After painting.		Length of period.	Amount.	Sugar.							
			Before ether.	During ether.	Time.	Percent- age.										
										per cent	per cent					hrs. min.
13	13.45	One-third (posterior free end). Both sides stripped.	2.0	0.10	0.11	4	0.12	}	18	4.4	Tr.	None before.	Ether blown through cone for 63 min.	38.6	Free end of pancreas put through a hole in rubber membrane which protected the viscera. Vessels leading to tip ligated. Each side painted six times.	
						→ 4	0.11		3	57	75	"	+		39.7	
	Male.					22	0.10		17	44	>140	0	+++		38.8	

14	8.75	One-seventh (posterior free end). Both sides stripped.	1.5	0.13	0.17	→	10 2 4 22	0.21 0.12 0.12 0.11	11 1 2 18	2.4 6.2 15.4 >100	Tr. 1.08 0 0	None before. + + + +	Ether blown through cone for 73 min.	38.9 38.2 39.4 39.5	No blood vessels tied. Pancreas brought outside peritoneal cavity as a loop and other viscera protected from ad-renalin flowing on them, by several layers of rubber tissue. Each side painted about seven times.
15	10.75	One-fifth (posterior free end). Both sides stripped.	1.5	0.11	0.13	→	10 2 4	0.15 0.11 0.11	11 2 1	1.8 44 60	0 0 0	None before. + + +	Ether blown through cone for 45 min.	38.4 38.6 38.5 39.2	The procedure same as in No. 14. Each side painted six or seven times.

TABLE IV—Continued.

Experiment No.	Weight. Sex.	Part and proportion of entire pancreas painted.	Amount of adrenalin (1:1,000 solution).				Blood sugar.				Urine.			Albuminuria.	Anesthesia.	Temperature of dog.	Remarks.
			Before ether.	During ether.	After painting.		Before ether.	During ether.	Time.	Percent- age.	Length of period.	Amount.	Sugar.				
	kg.		cc.	per cent	hrs. min.	per cent	hrs. min.	per cent	hrs. min.	cc.	per cent						
16	12.0	One-fifth (posterior free end). Both sides stripped.	1.5	0.12	0.15	→	11	0.21	13	1.4	0			None before.	Ether blown through cone for 64 min.	38.7	The procedure same as in No. 14. Each side painted three times.
	Male.				4	52	0.12		1 52	10	Tr.			+		37.9	
									2 36	27	0			+		38.8	
17	11.5	One-third (posterior free end). Stripped very little.	1.5	0.09	0.18	→	10	0.20	14	1.2	About 1			None before.	Ether blown through cone for 58 min.	39.5	The procedure same as in No. 14. Each side painted four or five times. Vomited during experiment. Pulmonary edema and pneumonia.
	Male.				1	24	0.13		58	9	2.66			+		38.5	
					19	25	0.13		18	>500	0			Tr.		39.0	

18	9.5 Male.	One-half (posterior free end and part of middle). Stripped well.	1.50.120.25	→	110.26 2 160.16 3 530.13 21 400.10	16 1 55 1 35 18	3 16.4 13 552	0.0 0.57 Tr. (?). 0.0	None before. + + + +	Ether blown through cone for 66 min.	39.2 37.4 38.3 37.0	Procedure similar to that for No. 14, except that the duodenum, wrapped in rubber tissue, was also brought outside. Each side painted three times. Dog dying next morning.
19	12.5 Male.	One-third (posterior free end and part of middle). Stripped very little.	2.00.120.21	→	140.25 3 170.12 5 100.12 23 300.15	33 50 1 52 18	4 60 55 642	About 0.7 Tr. 0.0 0.0	+ before. + + Tr. “	Ether blown through cone for 73 min.	37.0 38.2 38.6 38.9	Procedure similar to that for No. 18, except that other abdominal viscera were still better protected. Each side painted four times.
20	11.35 Male.	One-half (posterior free end and part of middle). Stripped well.	2.00.130.20	→	100.27 2 390.12 4 440.10 22 400.10	21 2 20 2 5 18	1.5 2.8 3.4 >1,000	0.0 0.0 0.0 0.0	+ before. Bloody. + + +	Ether blown through cone for 55 min.	36.1 38.1 37.3	Procedure similar to that for No. 19. Each side painted four times.

TABLE V.
Pancreas Isolated; Adrenalin Administered Intraperitoneally.

Experiment No.	Weight. Sex.	Blood sugar.				Urine.		Anesthesia.	Remarks.	
		Before injection.		After injection.						
		Before ether.	During ether.	Time.	Percent- age.	Amount.	Sugar.			
	kg.	cc.	per cent	hrs. min.	per cent	cc.	per cent			
21	11.5 Female.	2.0	0.12	0.26	15	0.32	1.4	About 1	Ether blown through cone for 3 hrs., 42 min.	Pancreas and duodenum lifted out of peritoneal cavity by loops of tape and wrapped in cloths soaked in warm saline solution, adrenalin injected below.
					35	0.29	9.8	5.33		
					1 30	0.23	8.4	5.33		
					2 40	0.22	3.4	6.00		
					3 40	0.16	4.0	2.85		
				→ 3 39	0.13	4.0	1.48			
22	9.3 Female.	2.0	0.12	0.23	15	0.27	0.8	0	Ether blown through cone for 3 hrs.	Procedure similar to that for No. 21. Vomited while under ether.
					46	0.27				
					1 46	0.28				
				→ 2 46	0.18	1.0	0			
23	10.35 Male.	2.0	0.11	0.18 30 min. later 0.15	15	0.16	5	Tr. 0.5 or less. 0.5 “ “	Ether blown through cone for 2 hrs., 7 min.	Procedure similar to that for No. 21.
					45	0.13				
					→ 1 47	0.12	A few drops.			
					2 47	0.11	1			

in glycosuria and hyperglycemia observed after intraperitoneal injections are not of pancreatic origin, the effects of the painting being much smaller when the organ is isolated from the peritoneum. This conclusion becomes even more evident when one examines the results of the few experiments given in Table V, in which adrenalin was injected intraperitoneally while the pancreas was isolated.

The rise in blood sugar in this table is: 0.06, 0.05, and 0.01 per cent, the average being 0.04 per cent. The glycosurias are: 6.0, 0.0, and 0.5 per cent, the average being 2.2 per cent. The number of these experiments is too small to permit of a definite decision, yet the conclusion is in harmony with that drawn from the experiments of Table IV; namely, that the hyperglycemia and glycosuria observed after intraperitoneal injections are not of pancreatic origin.

For the sake of clearness the averages of the amounts of urine and of blood sugar noted in Tables I to V are presented in Table VI.

TABLE VI.

Averages of Glycosuria and Rise of Blood Sugar Due to the Application of Adrenalin.

Averages.	Mode of application.	Glycosuria.	Blood sugar.
		<i>per cent</i>	<i>per cent</i>
Our 12 experiments (Tables I and II).	Painting unisolated pancreas.	2.23	0.054
Our 8 experiments (Table IV).	Painting isolated pancreas.	0.63	0.035
Our 3 experiments (Table V).	Intraperitoneal injections; pancreas isolated.	2.2	0.04
Vosburgh and Richards' 5 experiments (Table III).	Painting unisolated pancreas.		0.158

DISCUSSION AND CONCLUSIONS.

After Blum's discovery of the production of glycosuria by the subcutaneous injection of adrenal extract, Herter has the merit of having found that injection of adrenalin into the peritoneal cavity also produces glycosuria; this is an undeniable fact. Concerning Herter's claim that intraperitoneal injection gives a higher degree of glycosuria

than subcutaneous or intravenous injection, we offer no comment since we have made no observations on the glycosuric effect of subcutaneous injection of adrenalin, while we have made only three experiments by intraperitoneal injection. The most we can predicate on the basis of the present experiments is that intraperitoneal injection of adrenalin produces a somewhat higher degree of glycosuria than could be anticipated. However, in an earlier study carried out several years ago⁶ we arrived at the conception that the more slowly adrenalin was absorbed from the tissues into the circulation, the greater was its glycosuric effect; hence an intramuscular injection, which in its effect is nearly equal to that of an intravenous injection, induced a glycosuria definitely smaller than that set up by a similar dose administered subcutaneously. Unless the absorption from the peritoneal cavity is shown to be different from the absorption from subcutaneous injections, there could be no reason to assume that the glycosuric effect of intraperitoneal injection is much greater than that of subcutaneous injection. We might add that our former experiments do not support Herter's view that subcutaneous injection of adrenalin yields only slight degrees of glycosuria, because it is largely oxidized before entering the circulation. A difference exists in the effects upon blood pressure and upon sugar production, depending upon the mode of administration of adrenalin. With regard to the sugar production, a subcutaneous injection has a definitely greater effect than an intravenous injection; with regard to the blood pressure effect, however, the opposite is true. Herter states that an intraperitoneal injection of adrenalin exerts a smaller effect upon blood pressure than an intravenous injection—a fact which Auer and Meltzer can confirm for the rabbit.⁷

Our experiments lead us to conclusions which do not conform to those of Herter. It will be recalled that Herter and his coworkers state first, that painting the pancreas causes a marked glycosuria and hyperglycemia, and, second, that the glycosuria and hyperglycemia produced by intraperitoneal injections are of pancreatic origin; that is, they are produced by the adrenalin's coming in contact with the pancreas. In our experiments tabulated in Table IV, in which the

⁶ Kleiner, I. S., and Meltzer, S. J., *J. Exp. Med.*, 1913, xviii, 190.

⁷ Auer, J., and Meltzer, S. J., unpublished observations.

pancreas was isolated from the rest of the peritoneal cavity, the glycosuria was about one-third, and the rise in blood sugar about two-thirds that obtained by painting the unisolated pancreas. Hence two facts may be deduced: first, that the painting of the isolated pancreas produces only mild glycosuria and hyperglycemia, and, second, that the greater production of sugar observed after the painting of the unisolated pancreas cannot be of pancreatic origin. Indeed, our experiments point rather to the conclusion that the larger production of sugar after painting the unisolated pancreas is due to the fact that a large part of the adrenalin escapes to the peritoneum. The last mentioned view is supported by the statement of Herter and Wakeman⁸ that "applications to the kidney are apt to yield more sugar than similar application to the liver, intestine, spleen, or brain, but the glycosuria is less marked than after the pancreas has been painted." Emerson and one of us had shown that a dissolved substance painted upon a kidney with an intact membrane is incapable of penetrating the membrane and affecting the kidney, or even incapable of entering the circulation, except when the solution escapes to other parts of the peritoneum.⁹ It was this observation which led to the suggestion that the effects observed by Herter of painting the pancreas might have been due to the escape of adrenalin to the celiac ganglion. This point has not been directly tested, but several experiments were performed in which the adrenals were painted with the effect on sugar production apparently as intense as that obtained by painting the unisolated pancreas. However this may be, and whether the production of sugar after painting the unisolated pancreas is due to the escape of adrenalin to some definite organ covered by the peritoneum (celiac ganglion or adrenals) or whether the peritoneum as a whole is responsible for the sugar production, it appears that, when sugar production follows the intraperitoneal injection of adrenalin, it is not of pancreatic origin.

⁸ Herter and Wakeman, *Tr. Assn. Am. Phys.*, 1902, xvii, 578, foot-note.

⁹ Emerson and Meltzer, S. J., cited in *Tr. Assn. Am. Phys.*, 1902, xvii, 595

THE RATE OF DIALYSIS OF THE BLOOD SUGAR IN EXPERIMENTAL DIABETES.*

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Dialysis methods have been used by many investigators in the attempt to determine whether the sugar in the blood is "free" or "combined;" that is, whether it is a crystalloid in simple solution or exists in some more or less stable union with another substance. If it is free it will pass through a membrane permeable to sugar but if it is combined, the colloidal molecule, presumably formed should be held back. The technique of these various investigations has differed in many particulars,—in the proportion of the volume of the blood to that of the saline solution, in the manner of preventing clotting, in the composition of the saline solution, in the duration of dialysis, temperature, type of membrane, etc. In two respects, however, practically all resemble each other.

First, all have used the blood of normal animals exclusively. This is probably because of the conception that since normal blood sugar does not pass the kidneys it is in a non-diffusible state. However, the property of being secreted by the kidney is not necessarily a criterion of diffusibility. Various colloidal substances when administered parenterally are quickly thrown out by the kidney; the higher polysaccharides¹ are good examples. On the other hand, one frequently observes hyperglycemia without glycosuria both in human and in experimental diabetes, which would indicate, according to the above hypothesis, the occurrence of combined sugar in some diabetic bloods. Therefore, it seems evident that if combined sugar occurs

* A preliminary report of this work was presented before the Society for Experimental Biology and Medicine, March 20, 1918.

¹ Mendel, L. B., and Mitchell, P. H., *Am. J. Physiol.*, 1905, xiv, 239.

there is as great a possibility of finding it in diabetic as in normal blood. The higher percentage of sugar in diabetic blood is an advantage from an analytical standpoint which will be readily appreciated.

Secondly, all previous workers have determined the sugar content only at the beginning and at the end of the dialyzing period, which has usually been either 24 or 48 hours. Most of them² have found that normal blood sugar is completely, or nearly completely, removed by a sufficiently long period of dialysis. Lépine and Boulud³ found no sugar dialysis in 2 hours under suitable conditions but lately Lépine⁴ has stated that certain blood samples may contain both "free" and "combined" sugar, both of which can be shown to dialyze; it must be noted however that Lépine's "combined" sugar is that which is split off by heating with tartaric acid. Consequently although it cannot be said that the question is settled, the preponderance of opinion is that the sugar dialyzes freely under these conditions.

Michaelis and Rona⁵ have pointed out, however, that if both free and combined sugar exist side by side in the blood they are probably in equilibrium with one another. When the blood is dialyzed the free sugar will pass out, thus destroying the equilibrium and forcing the decomposition of a part of the combined sugar. The new free sugar formed will now dialyze and thus eventually as much sugar will be lost as if all the sugar originally present had been free. Therefore the complete disappearance of sugar from a dialyzing specimen would be no safe evidence that the sugar had not been, in part at least, in a non-diffusible state. They have accordingly dialyzed blood against varying percentages of dextrose dissolved in saline and have

² Schenck, F., *Arch. ges. Physiol.*, 1890, xlvii, 621. Arthus, M., *Z. Biol.*, 1896, xxxiv, 432. Asher, L., and Rosenfeld, R., *Biochem. Z.*, 1907, iii, 335. Edie, E. S., and Spence, D., *Biochem. J.*, 1907, ii, 103. von Hess, C. L., and McGuigan, H., *J. Pharmacol. and Exp. Therap.*, 1914-15, vi, 45. For a general discussion see Allen, F. M., *Studies concerning glycosuria and diabetes*, Boston, 1913, 284-290.

³ Lépine, R., and Boulud, *Compt. rend. Acad.*, 1906, cxliii, 539. Lépine, R., *Le diabète sucré*, Paris, 1909, 74.

⁴ Lépine, *J. physiol. et path. gén.*, 1917, xvii, 377.

⁵ Michaelis, L., and Rona, P., *Biochem. Z.*, 1908, xiv, 476.

found that the sugar content of the dialyzing blood remained constant only when the percentage of sugar in the saline was approximately equal to that in the blood. This is interpreted by them as indicating that the normal blood sugar is free.

EXPERIMENTAL.

Preliminary experiments with diabetic blood showed that determinations of sugar before and after a 24 hour dialysis gave no clearer evidence than the similar investigations mentioned above with normal blood. Diabetic blood and normal blood with added dextrose, when dialyzed against Ringer's solution for 24 hours in the cold, showed practically the same proportionate loss of sugar. It was then decided to use a shorter period and to take samples at frequent intervals. In the first experiments in which this was done artificial parchment thimbles were used. The results were very striking but a change had to be made in the type of dialysis membrane because the thimbles soon became difficult to obtain and, when obtained, were often found to be useless because of leaks. After considerable search a suitable type of membrane was found in the prepared mucosa of the sheep's cecum, used commercially for capping bottles of toilet preparations, etc. The various membranes of a single lot are fairly uniform in thickness and size, and in every case recorded below, the two or more dialyses, constituting a complete experiment, were performed with membranes of the same lot. All of the experiments about to be described were performed with this type of animal parchment membrane.

Method.

Experimental diabetes was produced in dogs by total or nearly total⁶ depancreatization. Blood was drawn from a cannula in a femoral artery or, usually, from a jugular vein either by cannula or by aspiration through a needle. When cannulas were introduced local anesthesia was used. The blood letting was done from 2 to 16 days after the operation. The blood was allowed to flow into a dry bottle or test-tube containing a weighed amount of dry hirudin, usually 8

⁶ Allen, Studies concerning glycosuria and diabetes, Boston, 1913, 476.

mg. of hirudin for about 20 cc. of blood. After stoppering, this was gently shaken for a few minutes. 15 cc. were then transferred to the dialyzing membrane. The latter had previously been soaking in Ringer's solution, after which the excess fluid was wiped off of both sides with filter paper, and it was then affixed to a suitable glass tube mounted in a clamp on a stand. The dialyzing membrane, containing the blood, was then immersed in 1 liter of Ringer's solution,⁷ until the surfaces of the blood and the Ringer's solution were at the same level. A 2 cc. sample was taken at this time for analysis from the residue of the 20 cc. of blood, and at hourly intervals from the dialyzing blood. Before each sampling the temperature of the outside fluid was observed and the blood was mixed as well as possible. After sampling, the dialyzing bag was adjusted so that the surfaces of the fluids would again be at the same level. The Lewis-Benedict method, as modified by Myers and Bailey,⁸ was used for the blood sugar estimations. In many of the experiments the system was kept at a low temperature by surrounding the jar with chopped ice and salt. In some, powdered thymol was added to both fluids.

As controls, normal dog blood was used. This was obtained and treated in exactly the same manner as the diabetic blood, except that weighed amounts of anhydrous dextrose were added in order to get a sugar content approximating the particular diabetic blood which it was desired to control. These controls were often shaken longer than the diabetic in order to be sure of solution of the dextrose; some even in a shaking machine to accomplish this. The control experiment was usually performed on the day after the diabetic and, of course, the attempt was made to have all experimental conditions the same.

RESULTS.

Chart I shows the types of curves which can be obtained when the blood is dialyzed against plain Ringer's solution. The diabetic

⁷ The Ringer's solution used had the following composition:

	<i>per cent</i>
NaHCO ₃	0.020
CaCl ₂	0.022
KCl.....	0.030
NaCl.....	0.800

⁸ Myers, V. C., and Bailey, C. V., *J. Biol. Chem.*, 1916, xxiv, 147.

blood sugar (Experiment D8' and D8'') dialyzes slightly slower than the control (Experiment D10' and D10'') during the 1st hour and very much slower during the 2nd hour. The 3rd and 4th hours show a

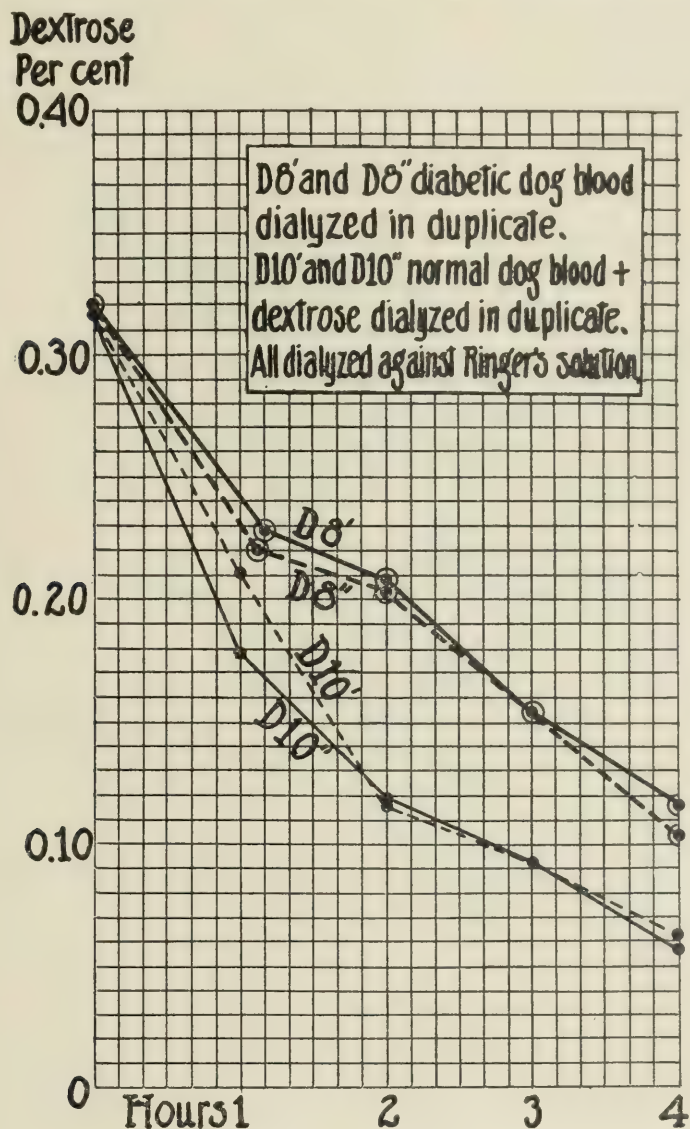


CHART I. Comparison of rate of dialysis of diabetic blood sugar with that of glucose dissolved in normal blood, using unmodified Ringer's solution. Arterial blood used. Room temperature: Experiment D8' and D8'', 20.5–23.2°; Experiment D10' and D10'', 20.0–22.2°. No thymol used. Diabetic blood from dog 2 days after complete pancreatectomy.

resumption of rapid dialysis on the part of the diabetic blood sugar. The control has a more rapid and more regular rate throughout, but

the most marked difference is in the 2nd hour in which the diabetic blood sugar dialysis is greatly retarded. It must be said, however, that such a marked difference is not always obtained when unmodified

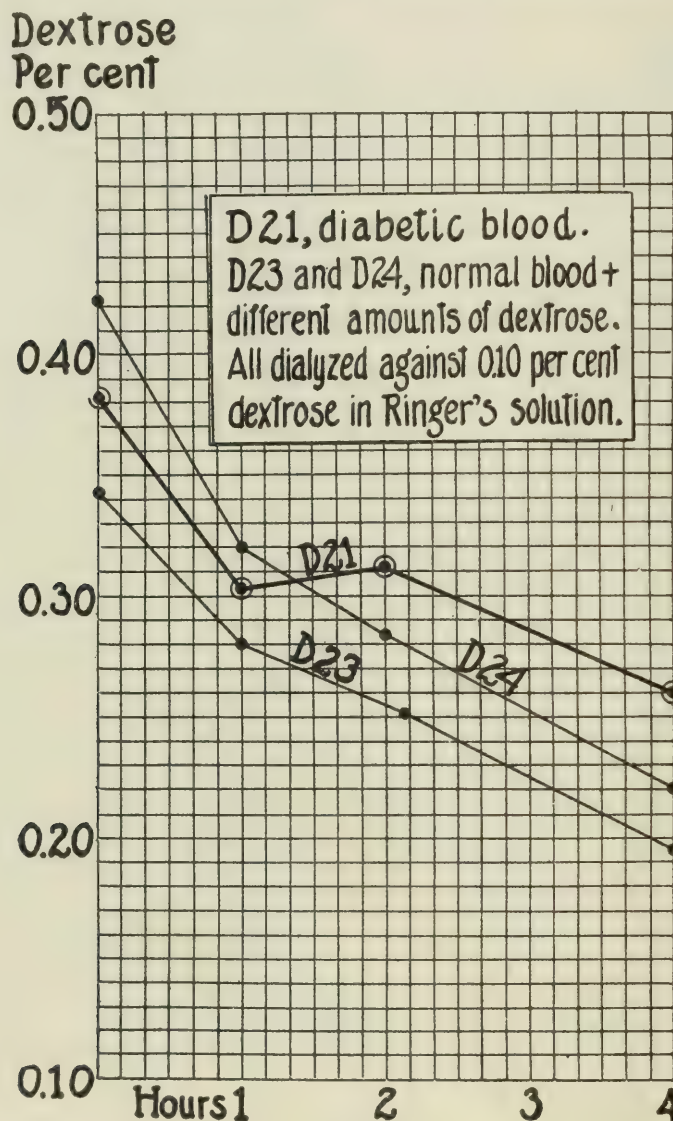


CHART II. Comparison of rate of dialysis of diabetic blood sugar with that of glucose dissolved in normal blood dialyzed against Ringer's solution containing 0.10 per cent dextrose. Venous blood used. Temperature: Experiment 21, 4.0–13.5°; Experiments 23 and 24, 3.0–12.0°. Powdered thymol added to blood and Ringer's solution in each case. Diabetic blood from dog 16 days after 95 per cent of the pancreas had been removed (Allen method).

Ringer's solution is used. Nevertheless this was quite similar to the type of curve which had been obtained in the above mentioned ex-

periments in which artificial parchment thimbles had been employed. It was therefore decided to change the experimental conditions in order to accentuate the difference between the diabetic blood and the control, if indeed any difference really existed. The fact that the animal membranes were much thinner than the artificial ones and permitted a more rapid dialysis, led to the suspicion that the

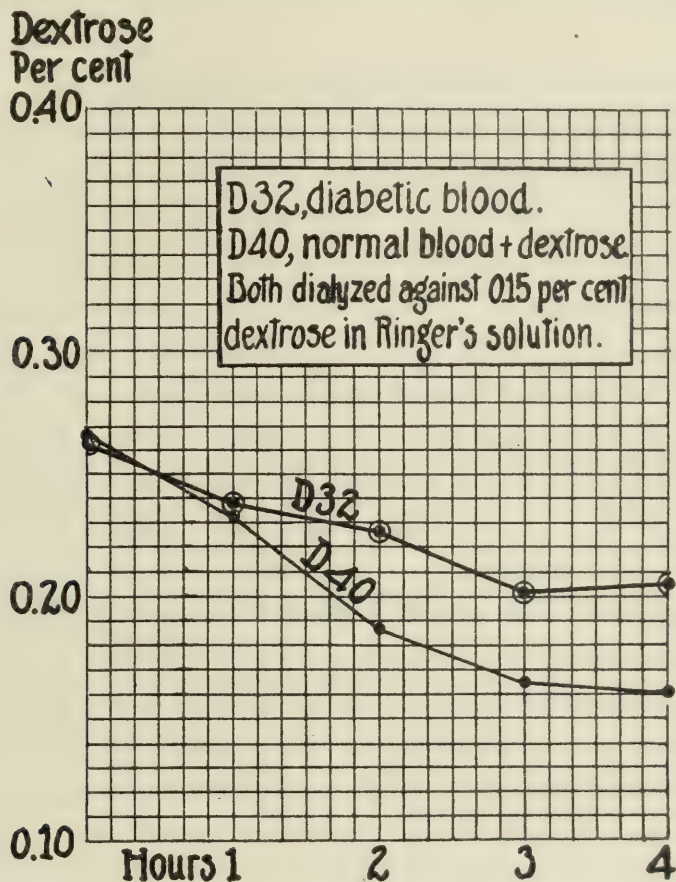


CHART III. Comparison of rate of dialysis of diabetic blood sugar with that of glucose dissolved in normal blood, dialyzed against Ringer's solution containing 0.15 per cent dextrose. Venous blood used. Temperature: Experiment 32, 1.3–2.0°; Experiment 40, 1.0–5.0°. No thymol used. Diabetic blood from dog 7 days after 95 per cent of the pancreas had been removed (Allen method).

production of a slightly slower rate of dialysis might accomplish this object. For this purpose a definite quantity of dextrose was added to the Ringer's solution in both the diabetic blood dialysis and the control. This modification of the procedure resulted in a retarded dialysis and was successful in bringing out very striking differences almost invariably.

The curves in Chart II tend plainly more to the horizontal than those of Chart I, indicating, in general, a slower rate. Here the Ringer's fluid contained 0.10 per cent dextrose. The two control curves show a continuous dialysis throughout the 4 hours. In the diabetic curve the dialysis runs parallel with the controls for 1 hour after which dialysis ceases completely for 1 hour. During the last 2 hours the sugar again passes out at a rate approximating that of the controls.

With a still slower rate a similar difference is observed. In Experiments 32 and 40 (Chart III) the blood was dialyzed against 0.15 per cent dextrose in Ringer's solution. Here again the control exhibits the same type of curve. The diabetic blood sugar dialyzes at the same rate as the control during the 1st and 3rd hours but is markedly retarded during the 2nd hour and does not dialyze at all during the 4th hour. This failure to dialyze during the 4th hour cannot be likened strictly to the slowing of the control curve during the same hour, because the latter has nearly reached the level of the outside fluid (0.15 per cent) while the former is still well above it. There are therefore two periods of delayed dialysis of the diabetic blood sugar in this instance.

More experiments were made with 0.10 per cent dextrose in the Ringer's solution than of any other type. Although Chart II is typical of them, the results of a number of others are given in Charts IV and V. These experiments differ with regard to temperature and some minor points in the technique, but the same type of membrane and outside fluid was used in each case. Chart V shows the diabetic curves, for which the curves of Chart IV served as controls. Five of the six diabetic curves exhibit a delay in dialysis during the 2nd hour; in one of these (D 36) dialysis is not resumed at all after this period. In the sixth curve (D 34) the delay is seen in the 1st hour and a good rate of dialysis thereafter. In Chart IV a regular rate of dialysis for the normal blood with dextrose added occurs in all cases as shown by the logarithmic type of curve or by nearly straight lines.

The average figures for Charts IV and V have been plotted and are given in Chart VI.⁹ Here the delay in the dialysis of diabetic

⁹ Several more experiments of this type have been performed since these curves were plotted but the results would not change the relative positions of the average curves.

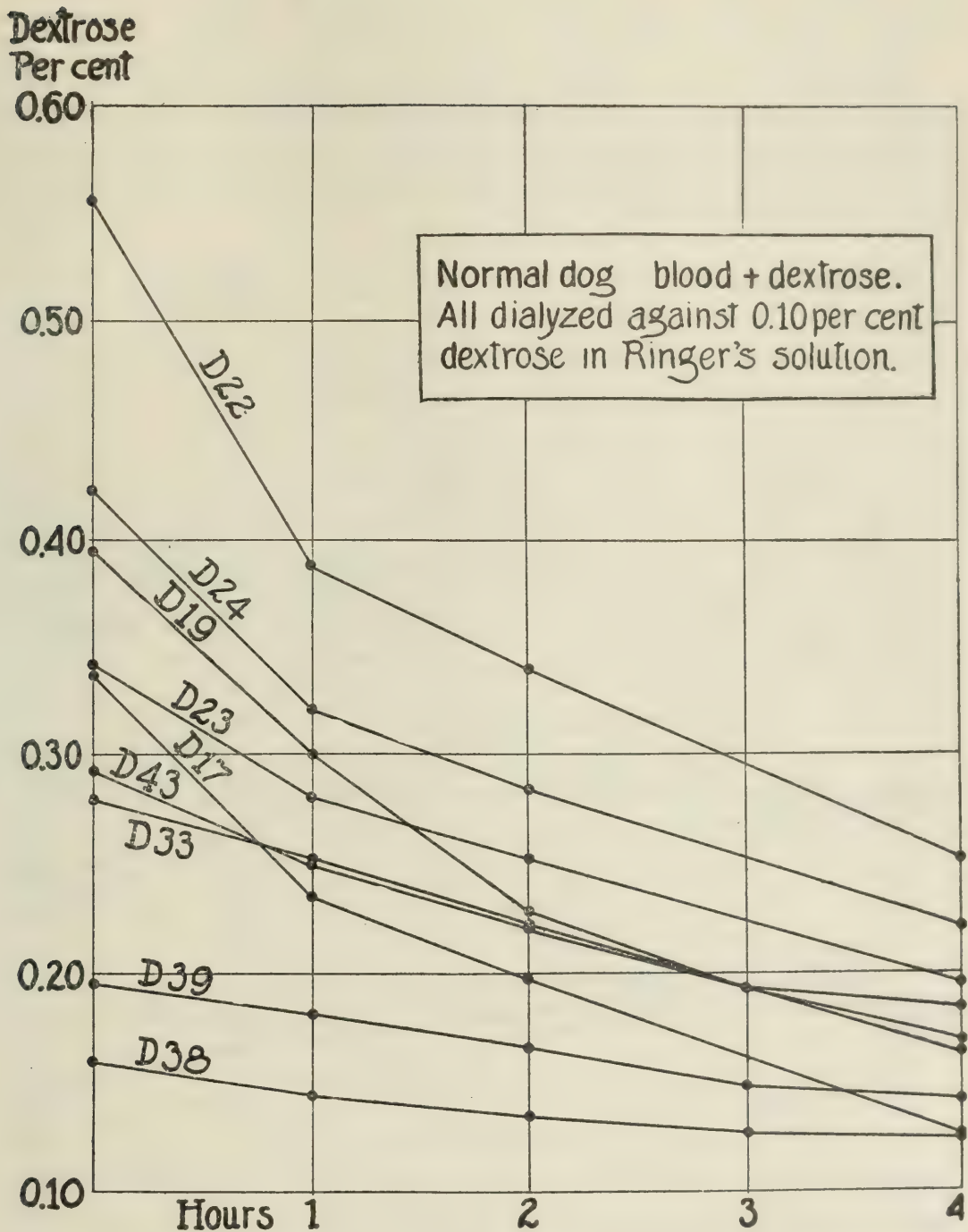


CHART IV. Dialysis curves of various specimens of normal blood with added dextrose, the temperatures varying, but all dialyzed against the same type of outside fluid.

blood sugar during the 2nd hour is clearly brought out. The control curve is a smooth, regular curve of the logarithmic type.

A few experiments have also been made with serum and with plasma. Although the results were not constant they seemed to indicate that the sugar in diabetic serum does not show any appreciable difference from the control. The diabetic plasma however, exhibited a slight retardation of sugar dialysis but not as clearly as the whole blood.

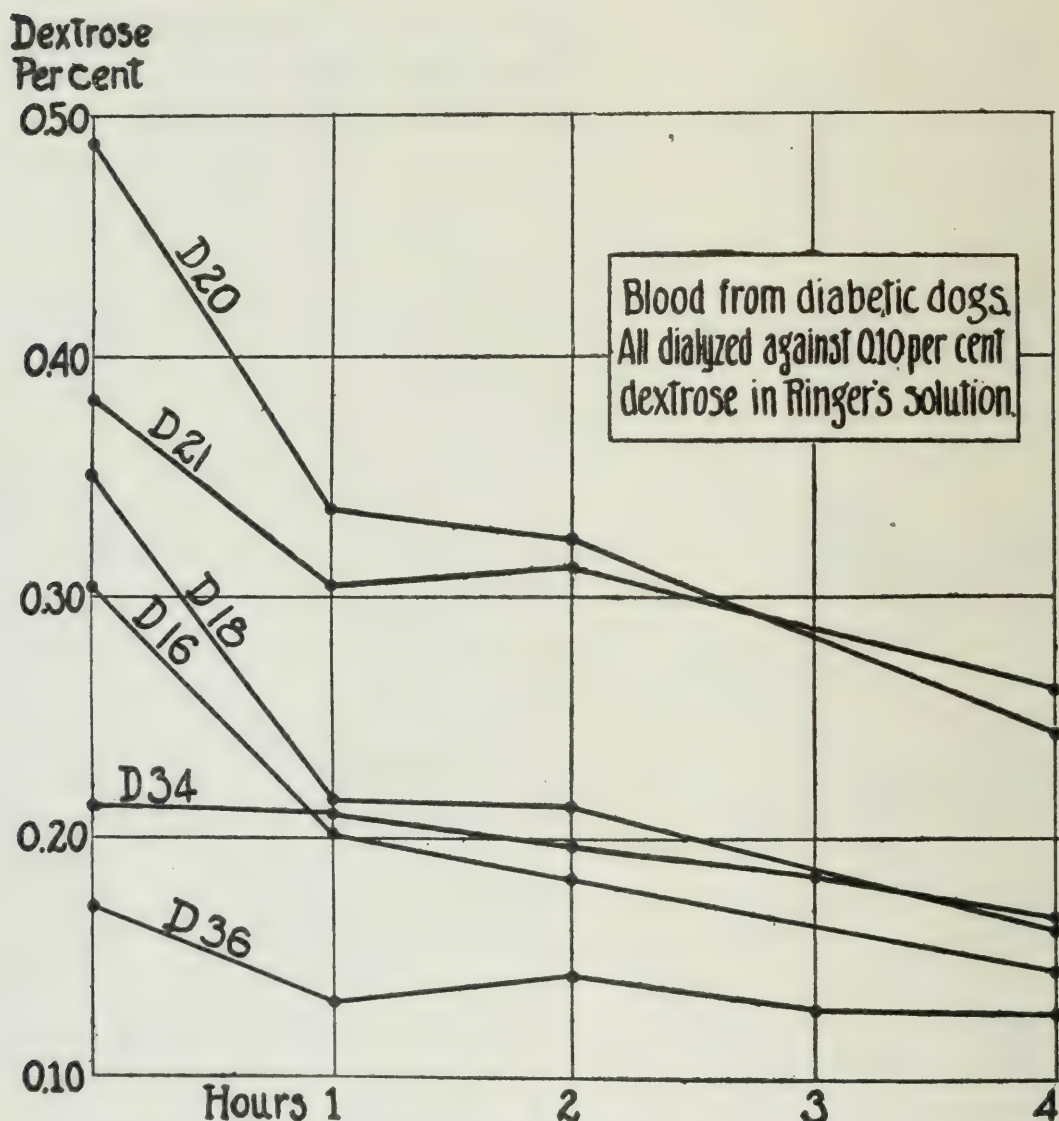


CHART V. Dialysis curves of various specimens of diabetic blood, the temperatures varying, but all dialyzed against the same type of outside fluid.

DISCUSSION.

The experiments described above show unmistakably that sugar does not dialyze from diabetic blood in the same manner as glucose which has been added to normal blood. There is a definite slowing, sometimes a complete cessation of dialysis at certain periods in the case of the diabetic as compared with the control. This is a definite

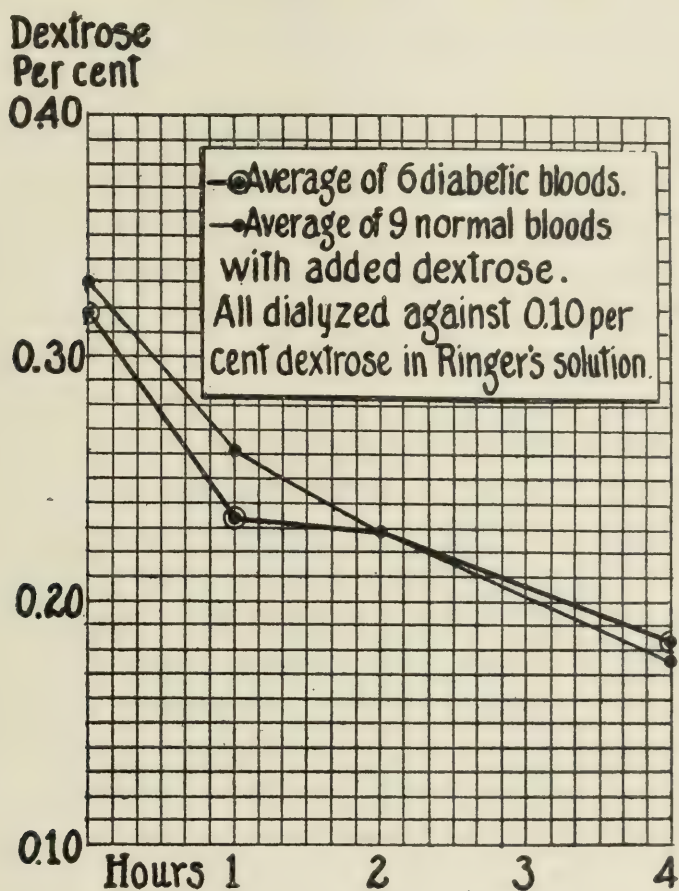


CHART VI. Dialysis curves representing the averages of the curves shown in Charts IV and V respectively.

fact and although it is easy to explain on the basis of the hypothesis which led to this investigation, several other interpretations can also be put forward.

In the first place the retardation may be due to a clogging of the membrane. It is well known, for example, that lipemia may occur after depancreatization. The fat or lipoids present in the diabetic

blood may perhaps form a film on the surface of the membrane or become dissolved in the membrane and thus inhibit the passage of the sugar through it. In order to produce a result like that of Chart I, for example, we must suppose that this film forms during or near the end of the 1st hour in order to prevent the dialysis during the 2nd hour and that it falls off or disappears after the 2nd hour in order to permit of the free dialysis which occurs later. Furthermore it would have to form again in order to give us the second delay seen in Chart III. Such changes, while not impossible, would seem to be unlikely.

There is also the possibility that these delays in dialysis are not delays at all but that the higher values obtained are due to a formation of sugar while the dialysis continues. In many of the experiments a sample of the blood was kept *in vitro* at the same temperature as the dialysis and during the same period. In no case did an increase in sugar content occur; in fact there was usually a slight decrease even at low temperatures. This is indeed no absolute proof that new sugar is not produced in the dialyzing specimen for here we might conceive of the removal, by dialysis, of substances which inhibit such production of sugar. In some of the experiments with diabetic blood there have been seen slight increases in sugar content in the course of the dialysis (see Charts II and V) but whether this is a real increase or is an error due partly to insufficient mixing before sampling, cannot be said. Lépine and Boulud³ found that after dialyzing fresh serum against saline for 2 hours the sugar content of the serum was often greater than that of the control kept at the same temperature. They explained this by assuming that a certain amount of water had passed through the membrane into the blood and had caused the formation there of dextrose from the *sucre virtuel*. Furthermore, Milne and Peters,¹⁰ as well as Myers and Killian¹¹ have shown that in depancreatized dogs the blood diastase is often greatly increased in amount. The formation of new sugar during the course of dialysis would seem then to be possible, and, from the evidence, more probable than an effect upon the membrane. However, the

¹⁰ Milne, L. S., and Peters, H. LeB., *J. Med. Research*, 1912, xxvi, 415.

¹¹ Myers, V. C., and Killian, J. A., *J. Biol. Chem.*, 1917, xxix, 179.

phenomenon described occurs to just as marked an extent—if not to a more marked extent—in the cold as at higher temperatures, a fact which would be inconsistent with an enzyme hypothesis. Moreover another one of the facts mentioned above cannot be explained on the basis of this hypothesis. This fact is that when the blood is dialyzed against unmodified Ringer's solution the difference between the diabetic blood sugar dialysis and the control is sometimes not well marked. If new sugar is being formed to any great extent it should, of course, regularly appear as an apparently retarded dialysis. In other words, the more rapid dialysis in this series, caused by a greater difference in osmotic pressure, should not have prevented a new formation of sugar. However, it did prevent the regular occurrence of a notable difference between the diabetic and control curves and this would seem to indicate that a new formation of sugar is not the chief cause of this phenomenon.

A third hypothesis is that part of the blood sugar in experimental diabetes is in a combined state. The type of combining substance or manner of combination need not be discussed, for such discussion would be mere speculation. According to this idea sugar is readily broken off from its combination by most chemical procedures; *e.g.*, as in the removal of the protein from the blood prior to sugar determination. Therefore the determination of blood sugar at any time during the dialysis reveals the amount of free and combined sugar present at that time. The combination is also easily split, probably, by physical means, such as the clotting of the blood, centrifugalization, and perhaps also by the action of an excess of water. Combined sugar, being a much larger molecule than free or crystalloid sugar, presumably does not pass through the membrane while crystalloid glucose easily does. If all the sugar in the blood were combined the dialysis curve would be a horizontal line, indicating no dialysis, until, from one cause or another, the combination would begin to break down, at which time the sugar liberated would begin to dialyze. If all the sugar is free the curves will be such as are seen in the controls of the present investigation.¹² The curves obtained with dia-

¹² The question of the occurrence of combined sugar in the normal blood of these controls may be disregarded for the purpose of this discussion because of the relatively large amount of crystalloid glucose added.

betic blood seem to indicate that the sugar is partly free and partly combined. It is true that it would be difficult to predict the exact form of curve which would delineate the dialysis of such a mixture. This is particularly true if the combined sugar is not in a stable union and may disintegrate either gradually or suddenly if the equilibrium between free and combined sugar is disturbed.

However, the curves obtained with diabetic blood lend themselves easily to interpretation from this view point. A large amount of free sugar—depending upon the proportion which is balanced by any sugar in the outside fluid—passes through the membrane first. The delay, which the curves show, whether in the 1st, 2nd, 3rd, or 4th hour, indicating as it does that dialysis is not continuing, means that there is now present a comparatively large proportion of combined sugar which cannot pass through the membrane. The resumption of dialysis is caused by a disintegration of the sugar combination, liberating free sugar which, of course, dialyzes. When there are two periods of retarded dialysis, as in Chart III (Experiment D 32), it would mean either that there were two types of combined sugar or that equilibrium was adjusted a second time.

The fact that with plain Ringer's solution the difference between the diabetic and control curves is not always sharp can be explained on the basis of this hypothesis as a sudden physical cleavage of the combined sugar molecule due to the extremely rapid dialysis, with a rapid upset of the equilibrium between free and combined sugar. With slower dialysis, produced by a thicker membrane, as in the preliminary experiments, or by decreasing the difference in osmotic pressure by adding dextrose to the Ringer's solution, the peculiar unevenly retarded curve has always been obtained with diabetic blood.

There is only one point which is difficult to explain by the combined sugar hypothesis; namely, the slight increase in the sugar which at times is seen in the course of the dialysis of a diabetic blood. If this increase is real, that is, if it is not an error of method or of sampling, there are two possible explanations, neither of which is entirely satisfactory. The first is that there may also be present a certain amount of another type of combined sugar which is precipitable by the protein precipitant (picric acid); and it is only when this combination disintegrates (and hence is not precipitated by the

picric acid) that its sugar appears in the analytical result as an apparent increase in sugar content. If this explanation is not acceptable then we must agree that there is active here, at the same time, a new formation of sugar to a slight degree. The possibility of a new formation of sugar as the explanation of the entire phenomenon has been discussed above as well as the reasons for considering this improbable. However, it may occur to a slight extent simultaneously with a delay in dialysis of combined sugar and thus give rise to the type of curve which has not only a delay but an increase in the blood sugar.

Significance.—Diabetes is a very complicated phenomenon and any new fact which can be established concerning it may aid in its explanation. The experiments described above indicate that *in experimental diabetes there exists some mechanism which tends to hinder the dialysis of the blood sugar*. Assuming that the same mechanism operates in the organism, can this fact aid in explaining diabetes?

For the production of hyperglycemia the presence of substances which can combine with glucose may, indeed, play some rôle. Let us suppose that such substances, whether toxic or not, are produced by the normal organism and are destroyed or neutralized if the pancreas is functioning in a normal manner. When the pancreas is removed, these substances circulate in the blood and combine with the dextrose. This leaves a dearth of free sugar in the blood and to remedy this condition the liver or muscles convert some of their glycogen store into dextrose. Thus we may have a hyperglycemia with varying proportions of free and combined sugar. It is also possible that the "combining substance" is toxic and that there is an outpouring of sugar in order to combine with and render it non-toxic; in other words this would be a protective action. However, whether primarily due to a sugar combination or to some other cause, hyperglycemia occurs. Why should hyperglycemia be maintained and why should the animal gradually lose its strength and waste away?

As a large proportion of the blood sugar is bound in the form of a colloidal molecule it cannot pass through the capillary walls, or does so with difficulty. Consequently it cannot reach the tissues and be utilized. It remains in the blood and thus the high blood

sugar is maintained, with usually a glycosuria as well. Moreover, the proportion of free dialyzable sugar is at the same time probably less than normal, and therefore, if other conditions remained constant there would be less free sugar available for the tissues, which would slowly starve. But the other conditions are not the same even for free sugar, for Kleiner and Meltzer¹³ have shown that when large quantities of glucose are injected intravenously, they pass into the tissues very slowly as compared with the normal, indicating a decreased permeability of the endothelium of the capillaries in diabetes. Therefore two factors tend to prevent the tissues from obtaining a supply of sugar; *viz.*, the conversion of a large proportion of the sugar into a difficultly diffusible form and the decrease in the permeability of the endothelium of the capillaries even to free sugar. The tissues in experimental diabetes apparently do not lack the normal capacity to burn or store sugar.^{10, 14} The fault is not that the tissues do not handle sugar well but that they cannot get the sugar. Palmer¹⁵ has shown that the striated muscle of diabetic animals has a lower concentration of dextrose than that of normal animals when the levels of blood sugar are taken into consideration. This fact tends to support the contention that, for one reason or another, the sugar does not get into diabetic tissues. Therefore, since the tissues are not properly nourished the animal gradually loses strength and ultimately dies.

The possibility that glucose combines with some substance or substances in the body has been suggested by various investigators. Several¹⁶ have believed that such a combination is a prerequisite for sugar utilization. Others¹⁷ have thought that this combination might be of such a nature as to resist oxidation. The present hypothesis,

¹³ Kleiner, I. S., and Meltzer, S. J., *Proc. Soc. Exp. Biol. and Med.*, 1914-15, xii, 58; *Proc. Nat. Acad. Sc.*, 1915, i, 338.

¹⁴ Cohnheim, O., *Z. physiol. Chem.*, 1903, xxxix, 341; 1906, xlvii, 253.

¹⁵ Palmer, W. W., *J. Biol. Chem.*, 1917, xxx, 79.

¹⁶ Pavy, F. W., *On Carbohydrate Metabolism*, London, 1906, 9, 33, 71, 111. Eppinger, H., and Falk, F., *Berl. klin. Woch.*, 1911, xlviii, 1625. Allen, *Studies concerning glycosuria and diabetes*, Boston, 1913, 402. Ringer, A. I., *J. Biol. Chem.*, 1914, xvii, 107.

¹⁷ Minkowski, O., *Arch. exp. Path. u. Pharmacol.*, 1892-93, xxxi, 179. Stiles, P. G., and Lusk, G., *Am. J. Physiol.*, 1903-04, x, 78.

which is merely an hypothesis on the basis of these dialysis experiments, presents a totally different conception; namely, that a considerable part of diabetic blood sugar, being in a combined and less easily diffusible condition, is not stored or used by the cells because it cannot pass from the blood through the walls of the capillaries and into the tissues.

The glycosuria of diabetes need not concern us in this discussion. It presents nothing which is incompatible with the present hypothesis, for, as stated above, the kidney is able to secrete colloids as well as crystalloids. Other questions in regard to the general problem have also been omitted for it is not to be expected that the observations recorded here will explain the entire question of diabetes. It is hoped, rather, that they will fit in with other facts and thus aid in its interpretation.

SUMMARY.

Blood from diabetic dogs was dialyzed against Ringer's solution, to which was usually added a small amount of dextrose. The rate of dialysis of the blood sugar was determined by analyzing samples taken at regular intervals. This was compared with similar dialyses of normal dog blood which had been brought to the same sugar content by the addition of dextrose. The diabetic blood sugar dialyzed at an irregular rate, with a delayed or completely interrupted dialysis during one or more periods, usually the 2nd hour. The control dialysis (normal blood with added sugar) was not interrupted in this manner.

This is interpreted as possible evidence for the existence of "combined" sugar in diabetic blood, the significance of which is discussed.

LECITHIN. II.

PREPARATION OF PURE LECITHIN; COMPOSITION AND STABILITY OF LECITHIN CADMIUM CHLORIDE.

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The preparation of pure lecithin has acquired in recent years an interest other than merely academic. The physiological action of lecithin has been studied from more than one point of view by several investigators. Yet it is safe to suspect that not one of them had the assurance of the purity of the material employed by him. Indeed, as it has been stated before, there is no record in the chemical literature on lecithin of a substance which answers all the requirements of a pure lecithin.

There exists a unanimity of opinion among workers on lecithin that cadmium chloride is the most serviceable reagent for the purification of the substance. This reagent was introduced many years ago and was more recently reintroduced for preparation of pure lecithin by Bergell.¹ However, at the time of Bergell's work it was not yet known that a sample of lecithin may possess the analytical values required by the theory for the pure substance and yet contain an appreciable proportion of cephalin.

The discovery that the base contained in cephalin was aminoethanol, indicated the way by which even small admixtures of cephalin could be detected in a sample of lecithin, the basic component of which is choline. The introduction by Van Slyke of a convenient method for amino nitrogen estimation has made the test for the presence of cephalin in a given sample of lecithin a comparatively easy task. Taking cognizance of this recently acquired information

¹ Bergell, P., *Ber. chem. Ges.*, 1900, xxxiii, 2584.

Maclean² made a new attempt to purify lecithin. He too made use of cadmium chloride for the precipitation of the phosphatide. By a series of purifications he arrived at a sample of the substance which was free from amino nitrogen, and which he considered a pure lecithin cadmium chloride salt. He also reached the conclusion that crude lecithin may be brought to a state of absolute purity by his procedure. The work of Maclean marks a great progress in the chemistry of lecithin. Unfortunately, on the one hand Maclean did not give complete analytical data of his purified material, and on the other hand very ingenious and careful workers such as Thudichum and later Erlandsen have found that samples of lecithin cadmium chloride, when reconverted into free lecithin, furnished material which no longer possessed the composition required by the theory for the pure substance. The conclusion of these workers was that in the process of liberation from the cadmium chloride salt, lecithin suffered partial decomposition. Indeed such a change might take place without having a marked influence on the numerical values of the nitrogen and phosphorus, since the proportion of these in lecithin is rather small.

Hence, the problem of lecithin as it presented itself to us in its latest phase was: First, is it possible to purify crude lecithin by means of cadmium chloride? Second, is it possible to reconvert a lecithin cadmium salt into free lecithin without its partial decomposition?

The experience in this laboratory in handling various tissue elements has brought the conviction that the majority of them did not possess the great lability ascribed to them by the earlier workers. In fact the greatest difficulty in unraveling the structure of these substances lay not in their lability but in their too great resistance to the action of degrading reagents. Hence, at the very outset we were inclined to accept the correctness of the observations of Thudichum³ and of Erlandsen⁴ as to the difficulty of obtaining an analytically true lecithin from a cadmium salt possessing correct analytical

² Maclean, H., *Biochem. J.*, 1915, ix, 351.

³ Thudichum, J. L. W., *The chemical constitution of the brain*, London, 1884.

⁴ Erlandsen, A., *Z. physiol. Chem.*, 1907, li, 116.

data. We were not ready to accept the interpretations offered by these workers. It seemed to us possible that the alteration in the elementary composition might have been brought about not by a cleavage but by a chemical alteration of the original material. Bearing in mind that cephalin is a frequent admixture of lecithin, and bearing in mind the readiness with which the latter undergoes alteration, we made the following assumption. The original cadmium salt in the hands of Thudichum and of Erlandsen contained an admixture of cephalin. In the course of the prolonged manipulations required for the removal of the cadmium chloride the cephalin undergoes alteration, which then affects the elementary composition of the material in the direction observed by the previous workers. This assumption was fully sustained by experiment.

Two samples of lecithin cadmium chloride were employed for the work. One contained 7.6 per cent of its nitrogen in form of amino nitrogen, the other was entirely free from amino nitrogen. Both were freed from cadmium chloride in the same manner. For convenience of analysis the two samples of free lecithin were converted into their dihydro derivatives. The first sample showed a carbon content of 63.3 per cent instead of 65.3 required by theory, whereas the second sample possessed correct analytical values. Thus it was conclusively demonstrated that it was possible to obtain pure free lecithin from its pure cadmium chloride salt.

The second part of the problem, dealing with the extent of purification which can be accomplished by means of cadmium chloride, was solved in the following manner. The alcoholic extract of egg yolk or of yolk powder served as starting material. One sample of lecithin was prepared from the phosphatides precipitated by means of acetone. The other was obtained by precipitating with a solution of cadmium chloride from the egg oil (mother liquor from the phosphatides, which was liberated from acetone). The former samples contained 7.6 per cent, the latter about 2 per cent of its nitrogen in the form of amino nitrogen. The first sample was then purified, following exactly the directions of Maclean, and led to a sample of cadmium salt which possessed 6.7 per cent of its nitrogen in form of amino nitrogen. The second was purified by recrystallization from ethyl acetate-ethyl alcohol and furnished a cadmium salt free from impurities.

Thus it seemed that samples of lecithin which could be freed from cephalin rapidly, before the latter underwent oxidation, led to analytically pure lecithin through recrystallization of its cadmium chloride salt; samples which contained a high proportion of cephalin could not be purified by means of the cadmium process.

SUMMARY.

For the preparation of free analytically pure lecithin the following procedure is recommended:

Precipitate lecithin from egg oil by means of a solution of cadmium chloride.

Purify the salt by recrystallization from ethyl acetate until free from amino nitrogen.

Liberate lecithin from the cadmium salt by means of ammonium carbonate.

The details of the procedure are given in the experimental part.

EXPERIMENTAL.

Cadmium Chloride Compound.

Composition of the Salt of Cadmium Chloride and Lecithin.—The composition of the cadmium chloride compound of lecithin has been reported by various investigators as follows:

	C	H	N	P	Cd
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Bergell				3.01	12.62
Thudichum			1.34	3.28	
Erlandsen.....	45.66	7.07	1.46	3.19	17.54
Strecker ⁵	50.75	8.38			13.07
Ulpiani and Selli ⁶	50.16	8.29	1.37	3.09	13.93
Heubner ⁷				3.50	14.9
Eppler ⁸	46.41	7.53	1.46	2.95	17.46
	47.42	7.65	1.50	3.01	16.29

⁵ Strecker, A., *Ann. Chem.*, 1868, cxlviii, 77.

⁶ Ulpiani, C., and Selli, G., *Gaz. chim. ital.*, 1902, xxxii, 466.

⁷ Heubner, W., *Arch. exp. Path. u. Pharm.*, 1908, lix, 420.

⁸ Eppler, J., *Z. physiol. Chem.*, 1913, lxxxvii, 243.

Our experience has shown that this salt is of variable composition, depending upon the character of the lecithin used for its preparation. A few of the many samples analyzed are reported below.

Primary Alcoholic Extract.—The cadmium chloride compound was prepared in the usual manner: An alcoholic solution of lecithin was precipitated with an excess of an alcoholic solution of cadmium chloride, saturated at room temperature. The precipitate, which soon settled out, was washed thoroughly with 95 per cent and then with absolute alcohol, either by decantation or on a filter, and finally with dry acetone. The dry salt was recrystallized from a mixture of 2 parts ethyl acetate and 1 part 80 per cent ethyl alcohol.^{9, 10} The composition of the salt was variable, as the following analyses show.

Sample No.	C	H	N	P	Cd
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
321	49.24	7.87	1.73	3.49	
323	51.73	8.08	1.68	3.23	
336	50.30	8.09	1.55	3.18	
338	49.26	7.68	1.52		
341			1.52	3.07	
352			1.71	3.16	
533			1.44	2.92	
534			1.67	3.18	
458			1.75	3.15	12.73

Primary Ethereal Extract.—The primary ethereal extract was separated in the usual way and the lecithin fraction prepared as above:

Sample No.	C	H	N	P
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
374	49.45	7.88	1.37	2.90

Secondary Alcoholic Extract.—Samples of the cadmium salt prepared in the usual manner from the lecithin fraction of the secondary alcoholic extract showed nearly the same composition as the above.

⁹ Willstätter, R., and Lüdecke, K., *Ber. chem. Ges.*, 1904, xxxvii, 3753.

¹⁰ Maclean, *Biochem. J.*, 1915, ix, 370.

Sample No.	C	H	N	P	Cd
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
353	50.37	7.23	1.55	3.08	13.10
369	50.43		1.50	3.15	
378	48.25	7.74	1.45	2.97	

Mother Liquor of Cadmium Salt.—The alcoholic mother liquor from the first precipitation of the cadmium chloride compound, upon concentration, gave a thick sirup, soluble in alcohol, from which acetone precipitated a gum-like substance. The sirup, diluted with alcohol, gave an abundant precipitate with alcoholic cadmium chloride. This had a composition similar to the first precipitate.

Sample No.	N	P	Cd	NH ₂ -N
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
503	1.65	3.17	13.01	11.00

Cadmium Salt from Egg Oil.—In view of the results of Fränkel and Bolaffio,¹¹ who claim to have isolated a phosphatide, as the cadmium salt, from the acetone-soluble fraction of egg yolk, which contained 1.84 per cent N and 0.51 per cent P, or a ratio of N:P = 8:1, it was of interest to determine the composition of the phosphorus-containing lipoids which remain dissolved in the egg fat, extracted by means of acetone. The following fractions of the acetone extract were prepared:

A. The concentrated acetone extract (egg oil), a semisolid mass at 0° (but which could not be filtered at 0°), was extracted with acetone, thus removing a considerable amount of acetone-insoluble lipoids. The acetone solution was concentrated and the resulting oil extracted with a large volume of 95 per cent alcohol at room temperature (I). The residue was again extracted with alcohol at room temperature (II). A third extraction removed little material which was precipitated by cadmium chloride and was therefore discarded.

B. Another sample of the same oil was first extracted with ether, in order that any water-soluble nitrogen-containing compounds might thus be removed (they would remain in the aqueous por-

¹¹ Fränkel, S., and Bolaffio, C., *Biochem. Z.*, 1908, ix, 45.

tion). The ether was removed on the steam bath and the resulting oil extracted with alcohol as before (III).

C. A third experiment started with an ethereal solution of egg lipoids, prepared by extracting fresh egg yolk with ether and concentrating the solution. This extract was precipitated with acetone, the acetone-ether solution concentrated, and the resulting oil extracted with alcohol (IV).

These four fractions were then precipitated with alcoholic cadmium chloride solution, and the salt was purified in the usual way. Analyses do not indicate a compound with $N:P = 8:1$, but rather a lecithin-like body.

Fraction.	Sample No.	C	H	N	P	Cd	NH ₂ -N
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
I	446			1.41	2.93		
	453			1.43	3.05	10.75	2.4
II	445			1.41	2.65		
III	461			1.41	2.93	10.65	
	505			1.41	3.00		1.4
IV	374	49.45	7.88	1.37	2.90		
	377			1.37	3.04	13.60	

Optical Activity of the Cadmium Chloride Salt.—After trying many solvents, it was found that the cadmium chloride salt could be dissolved in boiling xylene, and that this solution, on cooling, could be used for determining the optical activity of the salt. Ulpiani and Willstätter and Lüdecke have shown that the activity of the glycerophosphoric acid of lecithin varies with the manner of the preparation of the material. Therefore the values obtained for the salt, which varied from $[\alpha]_D^{20} = +2.0^\circ$ to $+4.25^\circ$, are not to be considered absolute values, nor are the variations due to impurities. A comparison of the amino nitrogen content and the optical activity shows that the content of amino nitrogen does not influence the rotation of the salt. The same appears to be true of the hydrolecithin.

Recrystallization of the Cadmium Salt.—Results of various investigators, especially Heubner, would indicate that the cadmium chloride

salt was unstable, and that it could not be recrystallized without marked decomposition. To determine this several samples were recrystallized repeatedly, the N and P contents being determined for each sample. Our results indicate that the salt is stable under these conditions.

Sample No.	C	H	N	P	Cd
Series I.					
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
323			1.68	3.23	
336			1.55	3.18	
342			1.61	3.16	
Series II.					
458			1.75	3.15	12.73
459			1.81	3.07	13.53
462			1.70	3.12	
463			1.63	3.22	13.36
493			1.64	3.13	14.02
Series III.					
478	48.57	8.58	1.84	2.87	12.65
491			1.84	2.97	12.40
495			1.83	3.11	12.38
502	48.49	8.44	1.77	3.05	13.42
Series IV.					
152		48.12	7.70	1.40	2.93
171		47.88	7.79	1.46	3.09
179		48.12	7.70	1.55	
213		47.78	7.68		

The mother liquor of the various fractions on concentration gave an ether-soluble substance, which could be precipitated by pouring into absolute alcohol. Upon analysis it showed the following composition:

Sample No.	N <i>per cent</i>	P <i>per cent</i>
504	1.65	2.95
516	1.70	2.61

Ether Extraction of the Cadmium Salt.—The finely powdered cadmium compound was suspended in 6 parts of ether and allowed to stand over night. The following morning the ether was decanted as far as possible and the insoluble salt filtered off and recrystallized from ethyl acetate-ethyl alcohol mixture.

Sample No.	C <i>per cent</i>	H <i>per cent</i>	N <i>per cent</i>	P <i>per cent</i>	Cd <i>per cent</i>
349	50.17	7.81	1.54	2.62	12.40
351	49.39	7.46	1.52	3.25	

A second experiment was made with the cadmium salt from the secondary alcoholic extract, using first ordinary ether (a) and then extracting with moist ether (b).

Sample No.	C <i>per cent</i>	H <i>per cent</i>	N <i>per cent</i>	P <i>per cent</i>	Cd <i>per cent</i>	NH ₂ -N <i>per cent</i>
(a) 409			1.49	3.11		
(b) 422	49.85	8.08	1.55	2.90	14.91	1.6

Decomposition of Cadmium Salt with Ammonium Carbonate.—The first question we attempted to answer was whether we could decompose the cadmium chloride compound with ammonium carbonate, according to Bergell, and obtain from the decomposition product a cadmium chloride salt with the same composition as the original.

25 gm. of recrystallized cadmium chloride compound (No. 2) were suspended in 250 cc. of 80 per cent alcohol, the alcohol was heated to boiling, and the mixture treated with finely powdered ammonium carbonate until the boiling mixture was alkaline to litmus. The cooled alcoholic solution was filtered, concentrated *in vacuo*, the residue taken up in alcohol, and precipitated with alcoholic cadmium chloride solution. The salt which separated was recrystallized from ethyl acetate-ethyl alcohol (No. 4).

A second sample (No. 234) was decomposed in the same manner, but before precipitating with cadmium chloride the lecithin was washed with water and precipitated with acetone (No. 235).

Sample No.	C	H	N	P	NH ₂ -N
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
2	47.35	8.05	1.53	3.25	
4	47.95	8.33	1.62	2.31	
30	46.85	7.94			
116	47.92	7.82			
234	48.61	7.88	1.67	2.88	
235	48.91	7.99			
237	48.99	7.93			7.6
243	47.36	7.59			6.7

The last experiment was carried out in order to determine whether the method recommended by Maclean would give us an amino-free product.

100 gm. of Sample 234 were suspended in 900 cc. of ether containing a trace of alcohol, and allowed to stand over night. The ether was then decanted as completely as possible and a fresh lot of ether added. The following day the material was filtered off and recrystallized from ethyl acetate and ethyl alcohol. This product was suspended in boiling 80 per cent alcohol and decomposed with ammonium carbonate. The filtered alcoholic solution was concentrated, the lecithin washed with water, and the alcoholic solution precipitated with cadmium chloride. Analysis of the amino content showed that the product contained nearly as much cephalin as the original material (7.6 and 6.7 per cent amino nitrogen).

The same material was used to determine whether or not recrystallization from ethyl acetate and ethyl alcohol would give a product free from cephalin. Again the result was a negative one.

These results would indicate that there is little decomposition in the above process.

The second question to determine was the elementary composition of the lecithin obtained upon the decomposition of the cadmium salt. As we have already mentioned in the introduction, the result depends upon the character of the material which is used.

The first experiment was carried out with material which contained a considerable amount of amino nitrogen. This was decomposed in the usual manner, the lecithin obtained washed with water, and the product reduced with hydrogen. The hydro derivative was then crystallized several times from methylethyl ketone.

Sample No.	C per cent	H per cent	P per cent
137	63.29	10.75	3.87

The second experiment was performed on material which was practically free of amino nitrogen. The procedure was similar to that described above. Two different samples of the hydro derivatives, after several crystallizations from methylethyl ketone gave the following figures on analysis.

	Calculated:	Found:	
C.....	65.37	65.22	64.92
H.....	11.23	10.70	10.97
N.....	1.74	2.02	2.22
P.....	3.84	3.85	3.89

These results indicate, as we have mentioned above, that it is possible to obtain a pure hydrolecithin from the product of the decomposition of a pure lecithin cadmium chloride.

Preparation of Pure Hydrolecithin.—The process by which we obtained our sample of pure hydrolecithin may be outlined as follows: The egg oil, obtained upon concentrating the acetone-alcohol solution after the greater portion of the lipoids had been precipitated out, was redissolved two or three times in dry acetone, thus removing most of the lipid material. This egg oil was then extracted with a large volume of hot alcohol, the alcoholic extract cooled to 0°, the solution decanted from the egg fat, concentrated, and the lipid mixture (mainly lecithin with a little cephalin) precipitated with cadmium chloride. This salt was recrystallized four times from the mixture of ethyl acetate and 80 per cent ethyl alcohol. It was then free of cephalin. The salt was then decomposed according to Bergell, the lecithin washed with water, precipitated with acetone (Maclean), and reduced as described in our first paper.¹² The analysis of the hydro body is given above.

¹² Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1918, xxxiii, 111.

SOME HYDANTOIN DERIVATIVES.

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Mendel and Dakin¹ were the first to emphasize the fact that the generally accepted structural formula for allantoin contained an asymmetric carbon atom, and, because of this, should be capable of existing in two stereoisomeric forms. However, all attempts to resolve allantoin into its optically active components have failed. On the basis of this, Dakin² formulated a structure for allantoin which may be regarded as a tautomeric form to the one generally accepted. Dakin³ further carried his analysis to substituted hydantoins and discovered the peculiar behavior of the optically active hydantoins towards alkali, which results in the formation of inactive forms. This reaction is remarkable in that the racemization proceeds with a much greater velocity than that observed for other substances. On the other hand, methylethylhydantoin could not be racemized.

Dakin has also made the observation that optically active amino-acids give rise to optically active hydantoins. This observation seems to be of considerable interest in connection with Dakin's theory of the structure of hydantoins. Since the active forms of the amino-acids give rise to active hydantoins, it is logical to assume that the *dl* forms of amino-acids, when first converted into hydantoins, should consist of a mixture of the *d* and the *l* forms. Further, it is logical to assume that substances consisting of such mixtures should differ in their physical properties from their tautomeric forms. It seemed of interest, therefore, to compare the physical properties—such as melting point, crystal form, refractive index—of the *dl* hydantoins and the hydantoins inactivated by alkali. Had such differences been found, Dakin's theory would have received

¹ Mendel, L. B., and Dakin, H. D., *J. Biol. Chem.*, 1909-10, vii, 153.

² Dakin, H. D., *J. Chem. Soc.*, 1915, cvii, 434.

³ Dakin, *Am. Chem. J.*, 1910, xlv, 48.

additional support. This consideration prompted the undertaking of the present investigation some years ago. It was found, however, that both the inactivated and the *dl* forms, in the majority of instances, did not differ in their melting points, and, roughly, in their crystal forms. At this time, it is impossible to undertake the work on the refractive indices and the work has been abandoned. It is published in its present form because of the additional data it contains on various hydantoins earlier described and because of the new hydantoins prepared during the course of this work.

	Melting point.	
	Given. °C.	Found. °C.
Hydantoin acid.	155, 160	179-180
<i>dl</i> -Methylhydantoin.	150	155-156
<i>dl</i> - α -Phenylureidopropionic acid.	168, 170	174
<i>dl</i> - α -Phenylmethylhydantoin.	172, 173	178
<i>d</i> - α -Phenylureidopropionic acid.		175
<i>d</i> - α -Phenylmethylhydantoin.		178
<i>d</i> - α -Naphthylureidopropionic acid.		198-200
<i>d</i> - α -Naphthylmethylhydantoin.		166
<i>dl</i> - α -Naphthylmethylhydantoin.		179-180
Inactivated naphthylmethylhydantoin.		179-180
α -Ureidobutyric acid.		184
Ureidodimethylacetic acid.	160	184
<i>dl</i> -Hydantoinacetic acid.	214, 225	228-229
<i>d</i> -Hydantoinpropionic acid.	179, 180	179-180
<i>dl</i> -Hydantoinpropionic "	167-169, 165	179-180
Inactivated hydantoinpropionic acid.	167-169	177-178
<i>dl</i> - <i>p</i> -Hydroxybenzylhydantoin.	158-160	158-160
Inactivated benzylhydantoin.	158-160	158-160
<i>l</i> -Hydroxybenzylhydantoin.	158-160	158-160

EXPERIMENTAL.

Hydantoic Acid.—7.5 gm. of glycoll, 8.1 gm. of potassium cyanate, and 20 cc. of water were heated on the water bath for 1 hour. The solution was filtered and concentrated hydrochloric acid added until the reaction was acid to Congo red. The acid which separated out on cooling was recrystallized from boiling water. Hydantoic acid decomposes at 179-180° (uncorrected); Diels and Heintzel⁴ give the decomposition point as 155°, Weidel and Roithner,⁵ as 153-154°, Griefs⁶ as 160°.

⁴ Diels, O., and Heintzel, H., *Ber. chem. Ges.*, 1905, xxxviii, 305.

⁵ Weidel, H., and Roithner, E., *Monatsh. Chem.*, 1896, xvii, 188.

⁶ Griefs, P., *Ber. chem. Ges.*, 1869, ii, 107.

Calculated for $C_3H_6O_3N_2$	N	23.73	
Found.....	N	23.62	23.54

Hydantoin.—Hydantoin was prepared according to the two methods of Harries and Weiss⁷ and their later statement,⁸ that the two products were identical, was confirmed. The melting point was found to be 218–220°, as given by Tafel and Reindl.⁹

Calculated for $C_3H_4O_2N_2$	N	28.00	
Found.....	N	27.80	27.82

dl- α -Methylhydantoin.—6 gm. of *dl- α -ureidopropionic acid* and 50 cc. of 10 per cent hydrochloric acid were boiled for 2 hours and the resulting solution was concentrated to a small volume. The *dl- α -methylhydantoin* which crystallized out melted at 155–156° after one recrystallization. Dakin found the melting point to be 150°.

Calculated for $C_4H_6O_2N_2$	N	24.60	
Found.....	N	24.47	

dl- α -Phenylureidopropionic Acid.—This acid was prepared by shaking 5 gm. of *dl-alanine* in 50 cc. of water containing 1.5 gm. of sodium hydroxide with 6.5 gm. of phenylisocyanate for 2 hours. The diphenylurea was filtered off and the solution acidified with dilute hydrochloric acid. The insoluble phenylureidopropionic acid separates out at once and may be recrystallized from a large volume of boiling water. *dl- α -Phenylureidopropionic acid* forms small iridescent plates which melt at 174° with decomposition. Kühn¹⁰ gives the melting point as 170°, Paal¹¹ as 168°.

Calculated for $C_{10}H_{12}O_3N_2$	N	13.46	
Found.....	N	13.47	

dl- α -Phenylmethylhydantoin.—When the above ureido acid is heated with 20 per cent hydrochloric acid for 1 hour, it yields *dl- α -phenylmethylhydantoin*. Upon cooling the solution, the hydantoin crystallizes in long, silky needles which melt at 178°. Recrystallization from water did not change the melting point. Mouneyrat¹² gives the melting point as 172–173°.

Calculated for $C_{10}H_{10}O_2N_2$	N	14.74	
Found.....	N	14.94	

⁷ Harries, C., and Weiss, M., *Ber. chem. Ges.*, 1900, xxxiii, 3418.

⁸ Harries and Weiss, *Ann. Chem.*, 1903, cccxxvii, 355.

⁹ Tafel, J., and Reindl, L., *Ber. chem. Ges.*, 1901, xxxiv, 3288.

¹⁰ Kühn, B., *Ber. chem. Ges.*, 1884, xvii, 2884.

¹¹ Paal, C., *Ber. chem. Ges.*, 1894, xxvii, 976.

¹² Mouneyrat, A., *Ber. chem. Ges.*, 1900, xxxiii, 2395.

d- α -Phenylureidopropionic Acid.—This acid was prepared in the same way as the inactive compound. Recrystallized from boiling water, it forms small silky needles which melt at 175° with decomposition.

0.1000 gm. substance neutralized 9.67 cc. 0.1 N HCl.

Calculated for C ₁₀ H ₁₂ O ₃ N ₂	N	13.46
Found.....	N	13.54

Rotation:

0.2741 gm. substance in 5.9286 gm. acetone gave a value for $[\alpha]$ of + 0.18°.

$$[\alpha]_D^{20} = \frac{5.9286 \times 0.18^\circ}{0.2741 \times 0.5} = + 7.78^\circ$$

d- α -Phenylmethylhydantoin.—Prepared by boiling the ureido acid with 20 per cent hydrochloric acid, the hydantoin crystallized from the hot acid solution in short pointed silky needles, which melt sharply at 178°. The melting point was not changed on recrystallization.

0.1000 gm. substance neutralized 10.37 cc. 0.1 N HCl.

Calculated for C ₁₀ N ₁₀ O ₂ N.....	N	14.74
Found.....	N	14.51

Rotation:

0.2700 gm. substance in 5.0410 gm. 0.5 N sodium hydroxide gave a value for $[\alpha]$ of + 0.06°.

0.2031 gm. substance in 5.0988 gm. acetone gave a value for $[\alpha]$ of - 0.20°.

$$[\alpha]_D^{20} = \frac{5.0410 \times 0.06^\circ}{0.2700 \times 0.5} = + 2.24^\circ \text{ (sodium hydroxide).}$$

$$[\alpha]_D^{20} = \frac{5.0988 \times - 0.20^\circ}{0.2031 \times 0.5} = - 10.04^\circ \text{ (acetone).}$$

dl- α -Naphthylmethylhydantoin. — Inactive α -naphthylureidopropionic acid^{13, 14} was boiled with a large excess of 20 per cent hydrochloric acid for 1 hour. A small part of the reaction product was in solution at the end and separated in long silky needles upon

¹³ Neuberg, C., and Rosenberg, E., *Biochem. Z.*, 1907, v, 456.

¹⁴ Gulewitsch, W., *Z. physiol. Chem.*, 1911, lxxiii, 434.

cooling. Most of the hydantoin, however, was in the form of an oil at the bottom of the flask and quickly solidified. Naphthylmethylhydantoin is very insoluble in boiling water but may be easily recrystallized from dilute alcohol. It melts at 179–180°.

0.1000 gm. substance neutralized 8.51 cc. 0.1 N HCl.

Calculated for $C_{14}H_{12}O_2N_2$N 12.07

Found.....N 11.91

d- α -Naphthylureidopropionic Acid.—5 gm. of alanine, 10 gm. of naphthylisocyanate, and 20 cc. of 10 per cent sodium hydroxide were shaken for several hours, the reaction product was filtered, and the filtrate acidified with dilute hydrochloric acid. The acid separates at once and may be recrystallized from a large volume of boiling water or from dilute alcohol. It forms short needles, which melt at 198–200° with decomposition.

0.1000 gm. substance neutralized 7.8 cc. 0.1 N HCl.

Calculated for $C_{14}H_{14}O_3N_2$N 11.20

Found.....N 10.95

Rotation:

0.1900 gm. substance in 7.229 gm. 0.5 N sodium hydroxide gave a value for $[\alpha]$ of + 0.05°.

$$[\alpha]_D^{20} = \frac{7.229 \times 0.05^\circ}{0.1900 \times 0.5} = + 3.80^\circ$$

d- α -Naphthylmethylhydantoin.—Prepared in the same way as the inactive compound and recrystallized from boiling water or dilute alcohol, the hydantoin melts at 166°. Repeated crystallization did not raise the melting point. The inactive ureido acid crystallizes very well from 95 per cent alcohol.

0.1000 gm. substance neutralized 8.51 cc. 0.1 N HCl.

Calculated for $C_{14}H_{12}O_2N_2$N 12.07

Found.....N 11.91

Rotation:

0.2504 gm. substance in 5.2367 gm. acetone gave a value for $[\alpha]$ of – 0.42°.

$$[\alpha]_D^{20} = \frac{5.2367 \times - 0.42^\circ}{0.2504 \times 0.5} = - 17.85^\circ$$

Two attempts were made to inactivate this hydantoin with normal sodium hydroxide. The first consisted in allowing the hydantoin to stand with an excess of sodium hydroxide for 2 days. At that time the rotation was zero. Because part of the compound was in the form of the sodium salt, it was warmed on the water bath before acidifying. The product which separated proved to be the inactive naphthylureidopropionic acid, decomposing at 199–200°, and yielded, when heated with 20 per cent hydrochloric acid, the inactive hydantoin, melting at 179–180°. In the second experiment the heating on the water bath was omitted but the result was the same, the *dl*-ureido acid. Lack of material prevented the testing of a weaker alkali or of a shorter time of inactivation.

α-Ureidobutyric Acid.—10 gm. of *α*-aminobutyric acid, 8 gm. of potassium cyanate, and 25 cc. of water were heated 1.5 hours on a boiling water bath. The product which separated out on acidification melted, with decomposition, at 184°, both before and after crystallization from boiling water.

0.1000 gm. substance neutralized 13.0 cc. 0.1 N HCl.

Calculated for $C_5H_{10}O_3N_2$	N	19.30
Found.....	N	19.32

Heated 1 hour with 10 per cent hydrochloric acid, the above acid gave *α*-ethylhydantoin, melting at 121–122°. Koenigs and Mylo¹⁵ found 118–120°.

Ureidodimethylacetic Acid.—10.3 gm. of *α*-aminoisobutyric acid, 8.1 gm. of potassium cyanate, and 25 cc. of water were heated on the water bath 1.5 hours and then acidified with concentrated hydrochloric acid. Twice recrystallized from boiling water, in which it is fairly insoluble, the acid melts at 184° with decomposition. Urech¹⁶ gives the decomposition point as 160° and states that it is rather easily soluble in water.

Calculated for $C_5H_{10}O_3N_2$	N	19.30
Found.....	N	19.08

¹⁵ Koenigs, E., and Mylo, B., *Ber. chem. Ges.*, 1908, xli, 4435.

¹⁶ Urech, F., *Ann. Chem.*, 1872, clxiv, 274.

Upon heating 1.5 hours with 10 per cent hydrochloric acid, the acid forms α -dimethylhydantoin, which melts at 175–176° as given by Urech and by Heilpern.¹⁷

Calculated for $C_5H_8O_2N_2$	N	21.87
Found.....	N	21.73

p-Hydroxybenzylhydantoin.—The hydantoin from *dl*-tyrosine (Kahlbaum) and from active tyrosine was prepared according to the directions of Dakin¹⁸ and the properties as given by him confirmed.

The active compound was then inactivated by allowing a solution in *N* sodium hydroxide to stand about 24 hours. Upon acidification and recrystallization of the inactivated hydantoin from boiling water, it forms prisms of the same appearance as the *dl*-hydantoin, and not plates, as observed by Dakin. The melting points of the three forms, the *dl*, the *d*, and the inactivated, were the same, 158–160°, as given by Dakin.

dl-Hydantoinacetic Acid.—5 gm. of aspartic acid, 6 gm. of potassium cyanate, and 25 cc. of water were heated about 5 hours on a water bath. The resulting sirup was taken up in 10 per cent hydrochloric acid, boiled for 15 minutes, and the solution concentrated to dryness. Twice recrystallized from a little water it melts at 228–229° with decomposition. Dakin¹⁹ gives the melting point of the inactive compound, obtained by the action of alkali upon the active acid, as 225–228°. Johnson and Guest,²⁰ who prepared it by the action of chloroacetic acid upon 2-thiohydantoin-4-acetic acid, give the melting point as 214–215°.

Calculated for $C_5H_6O_4N_2$	N	17.70
Found.....	N	17.51

dl-Hydantoinpropionic Acid.—10 gm. of *dl*-glutamic acid, 7 gm. of potassium cyanate, and 25 cc. of water were heated on a water bath for several hours until a thick sirup resulted. This was then taken up in an excess 10 per cent hydrochloric acid and boiled 20

¹⁷ Heilpern, J., *Monatsh. Chem.*, 1896, xvii, 237.

¹⁸ Dakin, *Am. Chem. J.*, 1910, xlv, 55.

¹⁹ Dakin, *Am. Chem. J.*, 1910, xlv, 58.

²⁰ Johnson, T. B., and Guest, H. H., *Am. Chem. J.*, 1912, xlvi, 109.

minutes. The reaction product was evaporated to dryness in vacuum and the residue recrystallized twice from water. The acid melted at 179–180°. A second preparation showed the same melting point. Dakin²¹ gives the melting point of the inactive product obtained by racemization of the active acid as 167–169°. Johnson and Guest²² prepared the acid by desulfurizing the thiohydantoin and found the melting point to be 165°.

Calculated for C ₆ H ₉ O ₄ N ₂	N	16.18
Found.....	N	16.20

The active acid melted at 179–180° as given by Dakin.

0.2160 gm. substance in 5.2593 gm. 0.5 N sodium hydroxide gave a value for $[\alpha]$ of -1.65° in a 0.5 dm. tube.

$$[\alpha]_D^{20} = \frac{5.2593 \times -1.65^\circ}{0.2160 \times 0.5} = -80.30^\circ$$

During the course of 6 hours this value fell from -1.65° to -1.32° . The rate of inactivation is much more rapid in N sodium hydroxide (Dakin). The melting point of this substance was found by Dakin to be 167–169°. Crystallized from water, the melting point was found to be 177–178°.

²¹ Dakin, *Am. Chem. J.*, 1910, xliv, 59.

²² Johnson and Guest, *Am. Chem. J.*, 1912, xlvii, 249.

THE STOICHIOMETRICAL CHARACTER OF THE ACTION OF NEUTRAL SALTS UPON THE SWELLING OF GELATIN.

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I.

It is generally believed that the reactions of colloids do not obey the stoichiometric laws of general chemistry. This viewpoint is illustrated clearly in the discussion of the action of neutral salts upon such physical qualities of proteins, as the swelling power of gelatin. The colloid chemists state that acetates, sulfates, tartrates, and citrates influence the swelling in the opposite sense from chlorides, bromides, and nitrates, the latter causing more swelling of the gelatin than distilled water, while the former cause a shrinking of the gelatin. Moreover, they claim that the relative efficiency of the anions of the same group varies in a definite order. The influence of the cations is stated to be negligible. In a previous paper the writer has already reported facts showing clearly that the effect of neutral salts upon the swelling of gelatin is of a stoichiometrical character.¹ Thus it was shown that all the univalent cations of neutral salts, Li, Na, K, and NH_4 , influence the swelling of gelatin at exactly the same concentration no matter what the nature of the anion; and the writer had also proved that only an unsuitable method was responsible for the erroneous statements of the colloid chemists concerning the influence of salts upon the swelling of gelatin.

The source of error into which the colloid chemists have fallen in this case is not difficult to understand. It happens that an adequate chemical reaction between neutral salts and proteins occurs only when the salt is added in excess, and it also happens that a

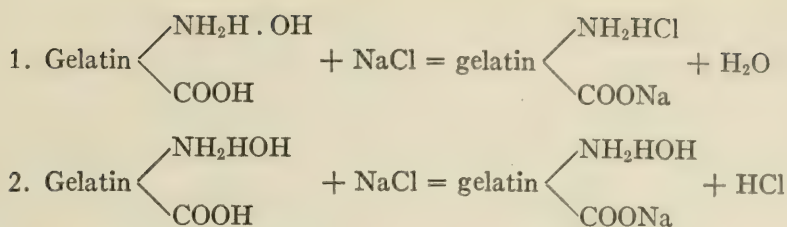
¹ Loeb, J., *J. Biol. Chem.*, 1918, xxxiii, 531.

number of the physical qualities of the new salt-protein compound only become apparent if the excess of the salt which acted upon the protein is afterwards washed away. In most previous experiments the effect of the salt upon the protein was tested in the presence of the salt; so that when little salt was used no or too small an effect on the protein was produced and when too much salt was used the salt present prevented the manifestation of the new qualities of the salt-protein product. Thus in Hofmeister's experiments² the swelling of a block of gelatin was measured in the presence of an excess of salt and he measured in reality the resultant of two opposite effects; namely, the mass action of the salt upon the protein and the inhibiting effect of the salt upon the swelling of the new salt-protein compound. It is no wonder that with such methods no stoichiometrical results were obtained.

Our method consisted in perfusing 2 gm. of finely powdered, non-bleached gelatin of equal size of grains (going through sieve size 50 but not through 60) twice with 25 cc. $M/8$ NaCl. It was assumed that with this excess of salt a certain percentage of the gelatin molecules would react with the NaCl and form a new compound. The gelatin molecules being amphoteric but stronger as acid than as base should form with the salt a compound which we will symbolize as Na-G (sodium gelatinate). When we remove the salt by perfusing the powder four times with 25 cc. of H_2O the Na-G formed is supposed to remain after the salt solution is washed away. Hence it is the additional swelling of this sodium gelatinate which served for the experiments in our previously published notes. - +

This compound dissociates electrolytically into $G + Na$, as was indicated by the fact that in the electrical field gelatin treated with NaCl, and then washed with H_2O , migrates exclusively to the anode (as does the gelatin not treated with salt, which is a stronger acid than base). This leaves the question open whether or not the Cl combines also with the gelatin or whether the reaction is only between Na and gelatin; in other words, Which of the two following possible reactions takes place?

² Hofmeister, F., *Arch. exp. Path. u. Pharm.*, 1891, xxviii, 210.



If the second type of reaction takes place, free acid, HCl, should appear in the supernatant salt solution. This was apparently not the case in our experiments. It should, however, be borne in mind that at least part of any free acid formed would combine with the gelatin and this combination between HCl and gelatin might occur

not only in molecules of the type gelatin $\begin{cases} \text{NH}_2\text{HOH} \\ \text{COONa} \end{cases}$ but also in the

molecules not in combination with Na; namely, gelatin $\begin{cases} \text{NH}_2\text{HOH} \\ \text{COOH} \end{cases}$

It is very probable that the reaction between gelatin and NaCl in our experiment is not complete; *i.e.*, does not transform all the gelatin molecules into Na gelatinate. If, therefore, part of the acid is caught by the gelatin, the fact that the reaction of the supernatant salt solution does not become markedly acid does not speak against the assumption that the reaction which occurs is the second reaction, which results in the formation of a sodium gelatinate. The decision of the question is not essential for our present purpose. We may state that when we treat gelatin with an excess of NaCl a compound is formed which dissociates electrolytically into Na and a negatively charged gelatin ion. If this gelatin ion contains also Cl the Cl is held in non-dissociable or less dissociable bondage; so that for all practical purposes we are dealing with a compound which we may call sodium gelatinate and which we will symbolize as Na-G.

Non-treated gelatin of the type gelatin $\begin{cases} \text{NH}_2\text{H.OH} \\ \text{COOH} \end{cases}$ swells in distilled water, but the swelling soon reaches its maximum. Sodium gelatinate swells also when perfused with an $m/8$ NaCl solution and reaches a maximum (which is lower than that of non-treated gelatin in H_2O). This swelling is the *initial* swelling which has

nothing or little to do with the effect of the salt and which will be disregarded by us. If we remove, however, the excessive salt by perfusing the powder repeatedly with a NaCl solution weaker than $M/8$ we find that below a definite concentration ($M/64$) of NaCl an *additional* swelling of the gelatin takes place, which becomes the greater the greater the dilution of the washing solution. This *additional* swelling which is the true effect of the salt upon the swelling of the gelatin we assumed to be due to the existence of the negative gelatin ion. That the gelatin ion has a negative charge in this case has nothing to do with the phenomenon of additional swelling, since positively charged gelatin ions (e.g., gelatin treated with acid) also give rise to an additional swelling. The assumption that the ionization of the gelatin is responsible for the *additional* swelling is not necessary for the proof of the stoichiometrical character of the influence of neutral salts on the swelling, though everything speaks in favor of this assumption.

The fact of importance for us is the following: An excess of NaCl is necessary to cause the formation of Na gelatinate and the *additional* swelling only begins to appear if the excess of the salt is removed and the $M/8$ NaCl solution is replaced by a $M/64$ NaCl solution. If the $M/8$ NaCl is replaced by a still weaker NaCl solution the additional swelling becomes still greater. *The stoichiometrical character of the inhibiting influence of neutral salts upon the additional swelling is revealed in the following facts.*

1. All neutral sodium salts with monovalent anion have under the conditions of the experiment the same limiting molecular concentration for the beginning of the additional swelling, namely $M/64$ (Table I, Group I).

2. All neutral sodium salts with bivalent anion have the same limiting molecular concentration for the beginning of the additional swelling which is $M/128$ —exactly half as large as that for the salts with monovalent anion. Since a $M/128$ solution of Na_2SO_4 has approximately as many sodium ions in solution as a $M/64$ NaCl solution, we can say that in all neutral solutions of a sodium salt with monovalent or bivalent anion the limiting concentration for the additional swelling is determined by the concentration of Na ions in the solution which is $M/64$ regardless of the anion (Table I, Group II).

3. The limiting concentration for the additional swelling remains the same when the salts treated first with $M/8$ NaCl are afterwards perfused with neutral solutions of salts of Li, K, and NH_4 with monovalent or bivalent anion (Table I, Groups I and II).

4. Non-electrolytes, like cane sugar, glycerol, alcohol, have no such limiting effect upon the additional swelling in concentrations of 2 M and below, but behave like distilled water (Table I, Group V).

These four facts are only comprehensible on the assumption that the additional swelling of gelatin depends upon the formation of well defined metal gelatinates in which the metal ions, Li, Na, K, and NH_4 , can replace each other in the same stoichiometric way as in the case of simple inorganic salts. The fact that the nature of both bivalent and univalent anions and univalent cations of the salt are of little if any importance fits well with the assumption that it is the ionization of the metal gelatinate which determines the additional swelling (Table I).

In order to understand the figures it should be stated that the perfusion of gelatin occurred in cylindrical funnels of the same diameter so that the increase in the height of the cylinder, expressed in the table in millimeters, was the measure of the swelling. The measurements were usually made after 24 hours to allow all the water which was not held by the gelatin or in the minute capillary spaces between the granules to run off.^{3, 1}

The perfusion of the 2 gm. of gelatin with 50 cc. $M/8$ NaCl causes a considerable initial swelling which does not increase when the perfusion with $M/8$ NaCl is repeated. When, however, for the subsequent perfusion (following that with $M/8$ NaCl) a weaker solution, namely $M/64$ or $M/128$ NaCl, is used, *additional* swelling takes place and this *additional* swelling is the true effect of the previous salt treatment upon the swelling of gelatin. As long as this additional swelling is less than 3 or 4 mm. in the height of the cylindrical mass of gelatin it may be disregarded since this is within the limits of unavoidable variation, due mainly to the formation of air bubbles in the mass and the error in measuring the height of the cylinder. The real additional swelling begins with an increase of 5 or 6 mm.

³ Loeb, *J. Biol. Chem.*, 1917, xxxi, 343.

TABLE I.
Action of Neutral Salts upon the Additional Swelling of Sodium Gelatin Produced by the Action of NaCl upon Gelatin.

Additional swelling of column of gelatin perfused twice with 25 cc. M/8 NaCl (to produce sodium gelatin) and three times with 25 cc. of the following solutions.

Type of salt.	Nature of salt.	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	M/4096	M/8192	H ₂ O
I. Univalent cation, univalent anion.	NaCl.....	0	0	2.5	6	15	21	33.5	42	48		mm.	54
	NaBr.....	0	2.0	2.0	5	12.5	17.5	27	43	42	46	53	52.5
	NaNO ₃	1	?	4	7	12.5	19.5	29.5	39	43.5	51	49	54
	Na acetate.....	1.5	1.5	3	6.5	15	22	31	38	45.5	44	48	55
	NaCNS.....		3.5	4	7	12	21	31	40	46	53	59	60
	LiCl.....	1	1.5	3.5	6	10.5	15	24.5	34.5	44.5	52	53.5	57.5
	KCl.....	1	2	2.5	6.5	11.5	16	24	33	38.5	44	46.5	53.5
	NH ₄ Cl.....	2	2	2	5	13	23	22.5	39.5	48	47		56
	Na ₂ SO ₄	1	1.5	2.5	3.5	8	14	22.5	30	39	47	55	54
	Na ₂ oxalate.....	2	2	2	3.5	6.5	14.5	24	29	35	46	50	56
II. Univalent cation, bivalent anion.	Na ₂ tartrate.....	2	2	2	3	5.5	13.5	23	32.5	43	46	52.5	56.5
	Na ₂ malate.....	0	1	1	2.5	5	13	20	31	35	39	43	51
	Na ₂ succinate.....		1	1	1.5	6	14.5	21	28.5	35	46	47.5	51.5
	Li ₂ SO ₄	3	0	2	2.5	3.0	10	19.5	30.5	45.5	51	51	60
	K ₂ SO ₄	1	1.5	0.5	3.0	6.5	11	20	29	40.5	42	50.5	54.5
	(NH ₄) ₂ SO ₄	1.5	1.5	2	3	6	12	18	26	42	50	45	53
	K ₂ oxalate.....	1	1	1.5	3.5	7.5	17	22.5	25	33.5	43	46.5	55
	(NH ₄) ₂ oxalate.....	0	1	1	2.5	5	11.5	22.5	27	36.5	48.5	49	54
	K ₂ tartrate.....	0	0	1.5	1.5	7	13	19	28	37	42	46	53
	(NH ₄) ₂ tartrate....	2	1.5	2	5?	7	16.5	22	35.5	43.5	49.5	52	55

III. Bivalent cation, univalent anion.	MgCl ₂	0	0	0	0	0	4	13	26	42	49	53	52
	CaCl ₂	0	0	0	0	0	1.5	8	24	39	48	51	47
	SrCl ₂	0	0	0	0	0	1	8	18	36	43	52	52
	BaCl ₂	0	0	0	0	0	1	10	21	40	45	51	50
IV. Bivalent cation, bivalent anion.	MgSO ₄	1.5	0	0	-1	0	1	7.5	22.5	38	48	55	63
	CaSO ₄					-3.5	-2.5	+8.5	18.5	37.5	43	47	55
V. Non-electrolytes.	Glycerol.....	43	42	42	40	41	41	41	43				42
	Cane sugar.....	51	51	50	50	51	51	51					48
	Ethyl alcohol.....	48.5	49.5	49.5	49.5	47	47.5	47					50.5

in the height of the cylindrical mass of gelatin. The figures in Table I give this increase in the height of the cylindrical mass of gelatin in millimeters. The reader will notice that the increase between 5 and 7 mm. lies at $M/64$ for all salts of Group I (type NaCl), while it lies at $M/128$ for all salts of Group II (type Na_2SO_4) regardless of the nature of the anion. This is the most fundamental of our results whereby the fact is established that we are dealing with simple chemical substitution phenomena.

Perfusion of Na gelatin with $M/128$ solutions of the salts of Group I (type NaCl) causes an additional swelling of from 10 to 15 mm. in the height of the cylindrical mass of gelatin; the same quantitative effect is found for concentrations of $M/256$ for salts of Group II (type Na_2SO_4). All through the table it is obvious that the salts of the type Na_2SO_4 give the same figures for additional swelling when their molecular concentration is one-half of that for Group I, type NaCl. This is true regardless of the nature of the anion or cation, as long as the latter belongs to the alkali group including NH_4 .

When we perfuse the gelatin (previously treated with $M/8$ NaCl) with solutions of non-electrolytes (sugar, glycerol, alcohol) of different concentration we get the same effect as when we use distilled water. This is one of the reasons why we are inclined to assume that the limiting concentration for the action of neutral salts upon swelling is that concentration which allows so many of the sodium gelatinate molecules to dissociate electrolytically that the additional swelling can begin. If our assumption is correct, the figures given in Table I might be utilized to calculate roughly the degree of electrolytic dissociation of the Na gelatin molecules in the solution of a salt of a given concentration, by taking the dissociation in H_2O as 100 per cent.

The reader will notice that we do not utilize the absolute amount of swelling as a criterion for our conclusions, but use only equal swelling in different concentrations of salt. The absolute swelling cannot be estimated by our figures since they include not only the water held inside the gelatin grains (the true swelling) but also the water held in the fine capillary spaces between the powdered granules which, however, may be only a comparatively small quantity.

It seemed desirable to see whether we could determine the limiting concentration for the swelling caused by neutral salts of Type I (NaCl) and Type II (Na_2SO_4) a little more accurately. For this purpose 2 gm. of powdered gelatin were perfused twice with 25 cc. $\text{M}/8$ NaCl and then with solutions from between $16 \text{ M}/512$ to $\text{M}/512$ of different salts of Types I and II. In this case we found again that the molecular concentrations of salts of Type I (NaCl) which just permit swelling are twice as great as those for salts of Type II (Na_2SO_4), and that the limiting concentrations are approximately between $3/512$ and $4/512$ for Salts II (Na_2SO_4) and between $3/256$ and $4/256$ for Salts I (NaCl) (Table II).

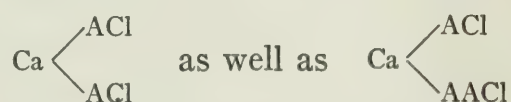
TABLE II.

More accurate determinations of the limiting concentration of different salt solutions upon the additional swelling of column of sodium gelatin.											
	8M/256	7M/256	6M/256	5M/256	4M/256	3M/256	5M/512	4M/512	3M/512	2M/512	M/512
	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.
NaCl.....	1.5	1	2	3.5	5	7		11.5		21.5	
NaNO ₃	1.5	3.5	2	2	3.5	8.5		13		23	
NaBr.....	2.5	2	3.5	3.5	5.5	8		14		24	
NaCNS.....	2	3	3	3.5	4	9		14		19.5	
LiCl.....	1.5	2	2	2	1.5?	5		10		18	
K acetate.....	2	1	4	4.5	3	10.5		16		26.5	
NH ₄ Br.....	1	0	2.5	3	4.5	7		14		22	
(NH ₄) ₂ SO ₄	1	1	0	0	1	1	2.5	4.5	8	13	21
Na ₂ malate.....	1		0	1	0	0.5	1	4	5.5	10	22.5
Na ₂ tartrate.....	1	1	1	1	0	1.5	3.5	5.5	9	16.5	22.5
Na ₂ oxalate.....	0	1	2	1	1	2	5.5	5.5	9	12	
K ₂ SO ₄					1.5	1.5	4	5	3	13	

From these facts it follows that only the cation inhibits the swelling in this case and that univalent cations, Li, Na, K, NH_4 , all act in exactly the same way in the same concentration no matter what the nature of the anion is. *We can, therefore, use the limiting concentration of a neutral salt for the additional swelling of sodium gelatin as a rough method for determining the molecular concentration of the salt and for this reason we are entitled to state that the action of the neutral salts upon the swelling of gelatin is of a strictly stoichiometrical character.*

In all the experiments thus far reported we always used the same ion-gelatin compound; namely, Na gelatinate prepared by treating the gelatin with $M/8$ NaCl. We have stated in a previous paper that we get the same results when we first treat the gelatin with some other neutral salt; *e.g.*, LiCl.

The effect of the neutral salts of the alkaline earth metals requires a special discussion. We have found that the salts of Mg, Ca, Sr, and Ba do not cause an additional swelling, and we are forced to the conclusion that such salts form either non-ionizable gelatin compounds or compounds of a different type from those which originate when salts of the alkali metals combine with gelatin. Pfeiffer and von Modelski⁴ have shown that Ca forms with amino-acids and betaines compounds containing two or three atoms of amino-acid in the molecule; namely,



The idea suggests itself that similar products originate when the metals of the alkaline earths combine with proteins like gelatin and that these larger compounds (of the type calcium gelatinate) do *not* cause any additional swelling. We will represent such compounds

under the symbol $\text{Ca} \begin{cases} \text{gelatin} \\ \text{gelatin} \end{cases}$ while the compound really formed may be of a more complicated or different nature than represented by our symbol and may also contain Cl. If it is the ionized gelatin which causes the additional swelling it follows that such compounds as calcium gelatinate do not dissociate electrolytically or if they do so their ionization will result in the formation of a polymerized or otherwise more complex gelatin anion, which for some unexplained reason is not capable of swelling.

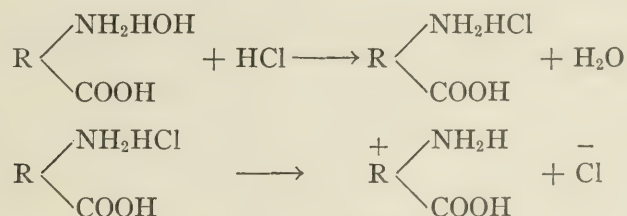
That the neutral salts MgCl_2 , MgSO_4 , CaCl_2 , CaSO_4 , SrCl_2 , and BaCl_2 , react with gelatin under the formation of definite metal gelatinates and that these compounds determine the negative influence upon swelling is indicated by the fact that the limiting concen-

⁴ Pfeiffer, P., and von Modelski, J., *Z. physiol. Chem.*, 1912, lxxxi, 329; 1913, lxxxv, 1.

tration is identical for all these salts; namely, about $m/512$ (Table I, Groups III and IV). This means that when we first perfuse gelatin with $m/8$ NaCl to form sodium gelatinate and then wash the NaCl away with solutions of salts of the alkaline earth metals, the additional swelling begins to appear when the concentration of the CaCl_2 , etc., is about $m/512$ or below. The fact that CaCl_2 and CaSO_4 , and MgCl_2 and MgSO_4 act alike confirms again the stoichiometrical character of the action of these salts on the gelatin.

II.

Hardy made the discovery that proteins when treated with acid migrate to the cathode and when treated with a base to the anode, and this fact was first interpreted as a "colloidal" phenomenon due to the "adsorption" of the H or HO ion by the protein⁵ or to the "transfer of the electric charges" of these ions to the protein. The writer pointed out that no such explanation was needed and that the phenomenon was a simple case of electrolytic dissociation due to the fact that the protein molecule is an amphoteric electrolyte, which in the presence of acid must dissociate as a base and become a cation, while in the presence of base it must dissociate as an acid and become an anion.⁶ This chemical view has since been generally accepted but with an important alteration suggested by Hardy that when acid is added to a protein a salt is formed which dissociates into the anion of the acid added and a positive protein ion.⁷



Therefore, if we treat gelatin with acid, *e.g.*, HCl, a salt (gelatin chloride) is formed which dissociates into a positively charged gelatin

⁵ The protein ion was supposed to be charged positively by the adsorbed H ion and negatively by the adsorbed HO ion.

⁶ Loeb, *University of California Publications, Physiology*, 1902-04, i, 149.

⁷ Hardy, W. B., *J. Physiol.*, 1905-06, xxxiii, 251.

ion and a negatively charged chlorion. When a 1 per cent solution is made of gelatin previously treated with HCl the gelatin no longer migrates to the anode but to the cathode.

This new compound, gelatin chloride, which dissociates into $\overset{+}{\text{gelatin}}$ and $\overset{-}{\text{Cl}}$ gives us an ideal chance to test our stoichiometric theory. If this theory is right we should expect that if we first treat powdered gelatin with dilute HCl, and follow this with three or four perfusions with various concentrations of neutral salts, we should expect the following results:

1. Neutral salts of the type NaCl (*e.g.* univalent anion, univalent cation) should permit additional swelling of gelatin chloride at the same degree of dilution which under the conditions of our experiments was again M/64.

2. Neutral salts of the type CaCl_2 (*i.e.* alkaline earth metals and univalent anion) should cause additional swelling of gelatin chloride in molecular concentrations exactly twice as dilute as salts of the type NaCl; namely, from M/128 down. The reader will notice that CaCl_2 should act upon gelatin chloride exactly as salts of the type Na_2SO_4 acted upon sodium gelatinate in the previous experiments reported in Table I.

3. Salts of the type Na_2SO_4 (*i.e.* univalent cation, bivalent anion) should allow additional swelling of gelatin chloride in concentrations from M/512 and below; in other words, Na_2SO_4 should act upon gelatin chloride as CaCl_2 did upon the sodium gelatinate in the previous experiments. It should form more complicated compounds symmetrical to those presumably formed by negative gelatin with

Ca, expressible in the symbol $\text{SO}_4 \begin{matrix} \swarrow \text{gelatin} \\ \searrow \text{gelatin} \end{matrix}$ corresponding to the symbol $\text{Ca} \begin{matrix} \swarrow \text{gelatin} \\ \searrow \text{gelatin} \end{matrix}$ discussed previously.

These predictions are fulfilled not only qualitatively but also quantitatively, as Table III shows.

In order to understand the results of this table it should be said that the preliminary perfusion with 50 cc. M/100 HCl caused a considerable swelling, due to the strong ionization of the gelatin chloride formed and the low concentration of the HCl solution used for per-

TABLE III.

Action of Neutral Salt upon the Additional Swelling of Gelatin Chloride Produced by the Action of HCl upon Gelatin,

Additional swelling of column of 2 gm. of powdered gelatin perfused twice with 25 cc. M/100 HCl (to produce gelatin chloride) perfused subsequently four times with 25 cc. of the following solutions.

Type of salt.	Nature of salt.	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	M/4096	H ₂ O
		mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.
I. Univalent cation, univalent anion.	NaCl.....	- 3.5	- 2	0	+ 4.5	12.5	21	23.5	27	30	31	31.5
	NaNO ₃	- 6	- 4	- 2.5	+ 4.5	10	17.5	25	31	33	29.5	38
	NaBr.....	- 8.5	- 3.5	- 2.5	+ 7.0	13.5	26	42	43	56	57	54
	NaCNS.....	- 7	- 5	- 2	0	+ 7.5	+ 13.5	26	25	29	27	33
	LiCl.....	- 6.5	- 5	- 1	+ 4	10.5	18.5	24	25	29	29	27
	KBr.....	- 6.5	- 4	+ 3.5	+ 6.5	15	20.5	35.5	33	50	41.5	37
	NH ₄ Br.....	- 8	- 5	0	+ 5.5	16.5	22	24	28.5	37	36	35
	Na acetate.....	- 7	- 5	- 2	- 2	- 4.5	0	+ 7.5	14	21	49	32
II. Univalent cation, bivalent anion.	Na ₂ SO ₄	- 8.5	- 8.5	- 10	- 8	- 4.5	+ 2.5	+ 20	+ 24	40	40.5	
	Na ₂ oxalate.....	- 7	- 8	- 10	- 7.5	- 9.5	- 7	+ 2.5	+ 22	22	26	89
	Na ₂ tartrate.....	- 4	- 6.5	- 7.5	- 8.5	- 6.0	+ 1.0	+ 3.5	+ 23	24	28.5	51
	Na ₂ malate.....	- 12	- 8	- 9.5	- 7.5	- 6.5	+ 1.5	- 4?	+ 16	36	47	50
	(NH ₄) ₂ SO ₄	- 6	- 8	- 6.5	- 5	- 3	0	+ 3	+ 16	23	24.5	35
III. Bivalent cation, univalent anion.	Mg(NO ₃) ₂	- 7	- 7.5	- 6	- 2.5	+ 4	+ 14	22	34	43	44	40
	CaBr ₂	- 9.5	- 8.5	- 8	- 3.5	+ 5.5	16	28.5	37	44	67	64.5
	Sr(NO ₃) ₂	- 6.5	- 7.5	- 5.0	- 3	+ 1.5	+ 8.5	16	27.5	30	35.5	39
	BaCl ₂	- 6.5	- 5.5	- 5.5	- 2.0	+ 4.0	+ 8.5	+ 19	31	32	34	60
	Sr acetate ₂	- 8.5	- 8	- 10	- 10.5	- 8.5	- 10	+ 9	+ 20	33	35.5	49
IV. Bivalent cation, bivalent anion.	MgSO ₄	- 7.0	- 7.5	- 4	- 2	- 5	0	+ 4.5	+ 13	26	31	38

fusion. When such a mass containing gelatin chloride was then perfused with a neutral salt above a certain critical concentration the ionization of the gelatin chloride was diminished and hence the gelatin gave off water and the swelling was reduced. Instead of causing an additional swelling the higher concentrations of the salt, $M/8$ or $M/16$, etc., cause a shrinkage indicated by the negative sign in the table. This shrinkage ceases with that degree of dilution of the salt solution which no longer diminishes the original degree of electrolytic dissociation of the gelatin chloride, and with the next degree of dilution the *additional* swelling of the gelatin begins, indicated in the table by a plus sign.

We have arranged the salts in the same groups as in Table I. The first group includes the salts with univalent cation and anion. The additional swelling of gelatin chloride begins in the same dilution (with the exception of Na acetate which will be discussed later) as was the case for the sodium gelatinate in Table I; namely, at $M/64$. Second, it can be seen that the additional swelling begins in Group III (type CaBr_2) for our gelatin chloride at exactly twice the critical dilution as for Group I; namely, at $M/128$. This proves that regardless of the nature of the cation the additional swelling begins at exactly the same concentration of the (univalent) anion, and the value is the same for different univalent anions, for the simple reason that if a gelatin chloride is treated with a nitrate, gelatin chloride is transformed into gelatin nitrate.

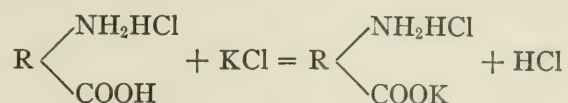
Finally, the bivalent anions behave toward gelatin chloride as do the bivalent cations toward sodium gelatinate; namely, they begin to permit additional swelling at a concentration of $M/512$. We assumed that this behavior was due to the formation of larger complexes of gelatin symbolized in the form $\text{SO}_4 \text{ gelatin}_2$ or $\text{SO}_4 \text{ gelatin}_3$, which apparently do not ionize.

The acetates, Na acetate as well as $\text{Sr} (\text{acetate})_2$, behave like the bivalent anions, for some unexplained reason possibly connected with hydrolytic dissociation.

Wherever shrinking effects of sulfates, oxalates, etc., upon the swelling of colloids actually occur, we are probably dealing with cases of protein salts of the type gelatin chloride, which are transformed into salts of the type of protein sulfate, this latter type being as incapable of swelling as salts of the type calcium gelatinate.

It might be argued that the statements of colloid chemists concerning the specific effects of anions are correct when applied to gelatin chloride. Thus the shrinking influence of the sulfates and acetates might be claimed as a confirmation of their views. Such a statement would overlook, first, the fact of the qualitatively and quantitatively identical action of sulfates, oxalates, tartrates, malates; second, of the identical action of Cl, Br, NO₃, CNS; and third, of the identical action of Li, Na, K, NH₄, Mg, Ca, Sr, and Ba. The truth is that the influence of neutral salts upon the additional swelling of gelatin chloride is essentially of a stoichiometrical character, though for some unknown reason acetate acts abnormally.

We have assumed that gelatin chloride reacts with neutral salts only by exchanging its anion. Hardy and Pauli⁸ have suggested a different reaction; namely,



If such a reaction takes place it is difficult to understand why CaCl₂ does not act in the same way upon gelatin chloride as it does upon sodium gelatinate; namely, by reducing its critical dilution (where additional swelling begins) to M/512, which is not the case. The fact that MgSO₄ behaves like Na₂SO₄ also suggests that only the anion of the neutral salt reacts with the gelatin chloride and that the cation does not participate in the reaction.

We, therefore, see that the limiting concentration of a neutral salt for the additional swelling of gelatin chloride could be used as a rough method for determining the molecular concentration of the salt. This is only another expression for the fact that the influence of neutral salts upon the swelling of gelatin is of a purely stoichiometrical character.

III.

When we treat gelatin with NaOH, sodium gelatinate is formed. This swells considerably, presumably on account of its high degree of electrolytic dissociation into a negative gelatin and a positive

⁸ Pauli, W., *Fortschr. naturwiss. Forschung*, 1912, iv, 223.

sodium ion. When we put such gelatin into a neutral salt solution that part of the swelling which is due to ionization of the protein (the "additional" swelling) should be suppressed as soon as the concentration of the salt solution reaches a certain critical value.

We assumed that when we treat gelatin with a neutral salt with univalent cation, NaCl or K_2SO_4 , sodium or potassium gelatinate is formed with a high ionization constant; while the anion of the neutral salt, Cl or SO_4 , does apparently not combine with the gelatin; or if it combines does not participate in the electrolytic dissociation. We have a chance to put this view to a test, since, if it is correct, gelatin treated previously with a neutral salt should behave like that treated previously with NaOH.

2 gm. of powdered gelatin were perfused twice with 25 cc. M/100 NaOH to form sodium gelatinate. The mass underwent a certain amount of swelling, the *initial* swelling, which will not be considered in the following figures. The mass was then perfused three times with 25 cc. of solution of different neutral salts, from M/8 to H_2O , to find out the limiting concentration where the different salts permit the beginning of the additional swelling. We notice that for Group I (type NaCl) this limiting concentration where the effect becomes positive is M/32; the salt with a weaker acid, Na acetate, and a weaker base, NH_4Br , forming exceptions in an opposite sense—possibly due to hydrolytic effects. The limiting concentrations for salts of Group II, type Na_2SO_4 , is one-half of the preceding, namely M/64; for salts of Type III ($CaCl_2$) and for Type IV it is M/256.

The relative values for these three groups are the same as for sodium gelatinate produced by the action of NaCl upon gelatin; namely, $NaCl:Na_2SO_4:CaCl_2 = 1:2:8$.

The absolute values for the limiting concentration of these different salts for additional swelling are about but not quite twice as high for the sodium gelatinate produced by NaOH as for sodium gelatinate produced by NaCl.

Theoretical Remarks.

Our experiments were carried on with two types of gelatin salts, sodium gelatinate and gelatin chloride. The former is produced by the action of NaOH or NaCl, the latter by the action of HCl upon gelatin.

TABLE IV.

Action of Neutral Salts upon the Swelling of Sodium Gelatin Produced by the Action of NaOH upon Gelatin.

Additional swelling of column of gelatin perfused twice with 25 cc. M/100 NaOH to produce sodium gelatin and then three times with 25 cc. of the following solutions												
Type of salt.	Nature of salt.	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	M/4096	H ₂ O
		mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.
I. Univalent cation, univalent anion.	KBr.....	- 3	- 2.5	+ 1	+ 9	+17	25	34.5	40	45	52	
	Na acetate.....	- 1.5	+ 1	+ 6.5	+11	16.5	21.5	29.5	39.5	41	42	39
	NH ₄ Br.....	- 4.5	- 8.0	- 2.0	+ 1.0	5.5	12.5	28.5	31.5	31.5	30.0	36
	LiCl.....	- 2.5	- 1.0	+ 3	10.5	12.0	51.0	55	64	59	46.6	63.5
	KCNS.....	- 2.5	0	+ 6.5	13	18.5	29	49	39	37.5		
	LiNO ₃	- 2	0	+ 3	+10.5	25.5	30.5	39	38	42	43	
II. Univalent cation, bivalent anion.	Na ₂ oxalate.....	- 2	- 1.5	+ 2.5	+ 7	+15	24	34	41	35	42	43
	K ₂ SO ₄	- 8	- 4	- 1	+ 4	+18	19	28	34	57	50	50
	K ₂ tartrate.....	- 5	- 9.5	+ 1.5	+ 6.5	+11.0	19.5	30.5	33.5	35.5	42.0	39.5
	Na ₂ succinate....	-12.0	- 5.5	- 3.5	+ 2.5	+10.5	20.5	20.5	33	35.5	35.5	35.5
III. Bivalent cation, univalent anion.	MgCl ₂	- 7	- 7	- 6	- 6	- 4.5	0	+11	19	28	29	59
	CaBr ₂	-12	-11	-10	-10	- 7	+ 3	+ 9.5	19	32	37	46
	SrCl ₂	- 8	- 6.5	- 6	- 2	- 2	- 2.5	+15.5	15.5	27	35	
	BaCl ₂	- 5	- 6	- 2.5	- 6.5	- 2	0	+13.5	16	27	30	47
IV. Bivalent cation, bivalent anion.	MgSO ₄	-13	-10	- 8.5	- 6.5	- 4.5	+10	19	29.5	40	45	62
	CaSO ₄					- 2.5	+ 2.5	11	23	32.5	40.5	40.5

The reaction between the type sodium gelatin and neutral salts takes place by the exchange of the cations; the anions of the neutral salts apparently not entering into the reaction with the gelatin. The reaction between gelatin chloride and neutral salts consists in an exchange between the anions, the cations apparently not participating in the reaction.

The gelatin compounds with univalent cation or anion, *e.g.* Na gelatin and gelatin chloride, are capable of swelling, while the gelatin compounds with bivalent anion or cation, *e.g.* Ca gelatin and gelatin sulfate, are not. The swelling of the former two types is apparently due to ionization and we may infer that the antagonistic effect of the Ca upon sodium gelatin and of SO_4 upon gelatin chloride is due to the formation of compounds not or less capable of ionization. Such an inference, though supported by certain facts, must, however, still be proved more directly before it can be accepted.

SUMMARY.

The new method described in previous publications for the determination of the effect of neutral salts upon the swelling of powdered gelatin has been applied to sodium gelatin prepared by the action of $\text{M}/100$ NaOH and to gelatin chloride prepared by the action of $\text{M}/100$ HCl upon gelatin.

1. It was found that the additional swelling of gelatin chloride, produced by the action of $\text{M}/100$ HCl upon gelatin, is inhibited in the solutions of neutral salts of univalent metals and univalent anions (type NaCl) in concentrations above $\text{M}/64$ and by salts with bivalent metal and univalent anions (type CaCl_2) in concentrations exactly half as high, namely above $\text{M}/128$, no matter what the nature of cation or anion. Salts with bivalent anion (type Na_2SO_4) have a limiting concentration of a much lower order; namely, $\text{M}/512$.

2. It was found that the additional swelling of sodium gelatin (produced by the action of $\text{M}/100$ NaOH upon gelatin) is inhibited in the solution of neutral salts of the type NaCl when the concentration is above between $\text{M}/32$ and $\text{M}/64$, and of salts of the type Na_2SO_4 when the concentration is half as high, namely above between $\text{M}/64$ and $\text{M}/128$, no matter what the nature of the cation or

anion. Salts with bivalent cation, type CaCl_2 , inhibit additional swelling in concentrations above between $M/256$ and $M/512$.

3. It was found that the additional swelling of sodium gelatinate formed by the action of high concentrations ($M/8$ or $M/4$) of NaCl upon gelatin is inhibited in the solutions of salts with univalent metal and univalent anion (type NaCl) in concentrations above $M/64$, and by salts with bivalent anion and univalent cation (type Na_2SO_4) in concentrations exactly half as high; namely, above $M/128$. The limiting concentration of salts with bivalent cation (type CaCl_2) is $M/512$. The neutral salts, therefore, produce the same type of compounds with gelatin as the bases; namely, metal gelatinates which dissociate into a positive metal and a negative gelatin ion.

4. These facts show that the limiting concentration of neutral salts for the additional swelling is within the restrictions mentioned independent of the nature of the anion and cation of the salt and that this limiting concentration upon the swelling of gelatin could be used to calculate roughly the molecular concentration of the salt used. The influence of neutral salts upon the swelling of gelatin is therefore of a stoichiometrical character.

5. When neutral salts act upon sodium gelatinate apparently only the cation of the salt combines with the gelatin; and when neutral salts react with gelatin chloride only the anion will combine with gelatin but not the cation. When neutral salts act upon ordinary gelatin, metal gelatinates are formed which may or may not contain the anion of the salt in non- or little dissociable bondage.

6. Our experiments show that gelatin salts with univalent anion (type gelatin chloride) or with univalent cation (type sodium gelatinate) are capable of additional swelling, while salts of gelatin with bivalent anion (type gelatin sulfate) and with bivalent cation (type calcium gelatinate) are not capable of additional swelling. It has been suggested in the previous paper that this swelling of gelatin salts with univalent cation or anion is due to a high degree of electrolytic dissociation, while the lack of the power of swelling of the gelatin salts with bivalent cation or anion is due to the low degree of electrolytic dissociation of these compounds.

7. Phenomena of antagonistic salt action can be produced by the transformation of protein salts of the type sodium gelatinate which

are capable of additional swelling into salts of the type calcium gelatin not capable of additional swelling; or by the transformation of protein salts of the type of gelatin chloride capable of additional swelling into salts of the type gelatin sulfate not capable of additional swelling.

THE LAW CONTROLLING THE QUANTITY AND RATE OF REGENERATION.

BY JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Communicated, March 18, 1918.)

1. It is well known that isolated pieces of a plant or a lower animal may regenerate into a whole organism again. In order to replace the current vague speculations concerning this phenomenon by a scientific theory in the sense of the physicist, quantitative experiments are required. The writer has for the past two years made such experiments which have led to a remarkably simple law controlling the quantity of regeneration in an isolated piece of an organism. This law can be expressed as follows: The mass of tissue regenerated by an isolated piece of an organism is under equal conditions and in equal time in direct proportion to the mass of growth material contained in the sap (or blood) of the isolated piece. The experiments on which this law is based were carried out on an organism unusually favorable for investigations of this kind, namely, the plant *Bryophyllum calycinum* (known to many laymen as the Bermuda 'life plant'). When leaves of this plant are isolated from the stem they will regenerate shoots in some or many of their notches. If a piece of stem is cut out from a plant it will form shoots from its two most apical buds. My experiments have yielded the result that the mass of shoots formed in the latter case is in direct proportion to the mass of a leaf attached to the stem; and to the mass of the isolated leaf in the former case. The data concerning regeneration in an isolated leaf have already been published¹ but will be repeated here to show the identity of the law in both cases.

2. When we cut out two sister leaves of *Bryophyllum*, *i.e.*, a pair of leaves taken from the same node of a plant, and keep them under the same condition of moisture, temperature, and light, the two sister leaves possessing equal mass will produce approximately equal

masses of shoots in equal times, although the number of shoots produced by the two sister leaves may vary considerably (table 1).

When we reduce the mass of one set of the sister leaves (by cutting away parts of the leaf), while that of the other set remains intact, both sets of leaves will produce in equal time and under equal conditions shoots whose masses are approximately proportional to the masses of the two sets of leaves (table 2).

From this it follows that equal masses of leaves produce equal masses of shoots, regardless of the number of shoots. Since chemical substances (water and solutes) are the only factors among those to be considered here which can vary in direct proportion with the mass

TABLE 1.

Influence of Mass of Leaves Upon Mass of Shoots Regenerated by Leaf.

		Weight of leaves.	Number of shoots.	Weight of shoots.	Milligrams of shoots produced per gram of leaf.
		grams		grams	
Experiment I. Duration, 22 days..	8 leaves	16.430	37	1.675	102
	8 sister leaves	16.476	40	1.682	102
Experiment II. Duration 29 days..	9 leaves	12.022	24	1.436	119
	9 sister leaves	11.861	20	1.348	114
Experiment III. Duration, 30 days..	12 leaves intact	18.435	25	2.884	156
	12 sister leaves. each cut into pieces	17.070	50	2.747	161

of the leaves, it follows that the quantity of shoot formation in an isolated leaf is determined by the quantity of certain material contained in the sap of the leaf. This material is probably the usual material required for growth: water, and certain solutes, sugar, amino acids, salts, etc.

3. It was necessary to test the validity of this law for the regeneration of shoots in isolated stems. The facts just mentioned suggested the method required to yield rational quantitative results. This method consisted in the measurement of the influence of the mass

of a leaf attached to a piece of stem upon the quantity of shoot formation in the latter. In order to obtain strictly comparable results, it was necessary again to compare the effect of sister leaves, since sister leaves alone are sufficiently alike to guarantee comparable results. The method of procedure was as follows. Stems of *Bryophyllum* containing three nodes and one pair of leaves in the third (most basal) node were split longitudinally into two halves, each half containing one leaf. One leaf remained intact, while the sister leaf attached to the other half of the stem was reduced in size by cutting away the greater part. Six whole stems were used for one experiment. After splitting, the halved stems were suspended in an aquarium with the

TABLE 2.

Influence of Mass of Leaves Upon Mass of Shoots Regenerated by Leaf.

		Weight of leaves.	Number of shoots.	Weight of shoots.	Milligrams of shoots produced per gram of leaf.
		grams		grams	
Experiment I. Dura- tion 37 days.....	5 leaves, with center cut out	7.610	11	0.755	99
	5 sister leaves, intact	13.800	9	1.405	101
Experiment II. Dura- tion, 25 days.....	7 leaves, with center cut out	9.899	21	1.213	122
	7 sister leaves, intact	16.935	25	1.995	118
Experiment III. Dura- tion, 32 days.....	9 leaves, with center cut out	10.522	22	2.292	218
	9 sister leaves, intact	17.852	30	3.430	192

apices of the leaves just dipping in water. Each half stem formed one new shoot from the apical bud and new roots at the base, but regeneration started earlier in the half stems with a whole leaf attached than in the half stems with a leaf reduced in size. After about five weeks the regenerated shoots were cut off and weighed. It was found that *the mass of the shoots regenerated in the two sets of halved stems was in exact proportion to the mass of the leaves attached to the stems* (table 3).

A similar law seems to hold for the root formation though this will have to be determined more definitely. The same law seems also

to hold for other cases of regeneration of *Bryophyllum* not discussed in this note.

We can, therefore, state that the quantity of regeneration in an isolated piece of an organism is under equal conditions and in equal time directly proportional to the mass of growth material circulating in the sap (or blood) of the piece and required for the synthetical processes giving rise to the regenerated tissues and organs. If we measure the rate of regeneration by the mass of material regenerated in a given time, the law expressed for the quantity holds also for the *rate* of regeneration and in this form the law becomes a special case of the law of chemical mass action.

4. This law does not throw any light upon two other features of

TABLE 3.

Influence of Mass of Leaves Upon Mass of Shoots Regenerated by Stem.

			Weight of leaves.	Weight of 6 regener- ated shoots on stem.	Milligram s of shoots regenerated per gram of leaf.
			grams	grams	
Experiment I. Dura- tion, 37 days.....	{	6 whole leaves	19.030	2.808	147
		6 sister leaves, reduced in size	2.853	0.443	152
Experiment II. Dura- tion, 34 days.....	{	6 whole leaves	18.490	3.586	192
		6 sister leaves, reduced in size	3.503	0.668	190

regeneration, namely, first, why is it that as a rule only the apical bud of an isolated piece of stem grows out and none of the buds situated more basally in the stem; and second, why it is that the same bud which grows out when the piece of stem is cut out from the whole plant does not grow out as long as the piece is part of a whole (and normal) plant. The writer published not long ago a series of experiments² which suggest that the growing apex (as well as the leaves) of a plant continually produce and send toward the base of the plant substances which *inhibit* the growth of dormant buds. When a piece of stem is cut out from a plant these inhibitory substances contained in the stem will continue to flow toward the base, with the result that the most apical buds will be the first to become compara-

tively free from these inhibitory substances and hence will be the first to grow out. As soon as this happens, the growing buds will produce and send toward the base inhibitory substances, with the result that none of the more basally situated buds of the piece of stem can grow out. In the normal plant the material serviceable for growth can only be utilized by the growing region at the apex (and the base of the plant); and when a piece is cut out from the plant the same material becomes available for the growth of those buds which are the first to be freed from the inhibitory material which they contained while forming parts of the whole plant. The further qualitative as well as quantitative experiments which the writer has carried out since the publication of his preliminary note support this hypothesis.

SUMMARY.

By measuring the influence of the mass of a leaf attached to an isolated piece of stem upon the process of regeneration in the piece, it has been possible to prove that the quantity of regeneration is in equal time and under equal conditions in direct proportion to the mass of the leaf. Since nothing except substances produced and sent out by the leaf can vary in direct proportion to its mass, it follows that the quantity of regeneration in an isolated piece of an organism is under equal conditions determined by the mass of material necessary for growth circulating in the sap (or blood) of the piece. If we measure the rate of regeneration by the mass of material regenerated in a given time, the law of regeneration becomes a special case of the law of chemical mass action. That this mass action on a bud is only possible in a piece of stem after it is isolated, the writer explains on the assumption that the apex of an intact plant sends constantly inhibitory substances into the stem preventing the buds contained in the stem from growing and consuming the material required for growth. When a piece of stem is isolated, the supply of these inhibitory substances from the growing region ceases and the most apical bud being the first to become free from the inhibitory substance will then come under the influence of the acting masses of the substances in the sap and regeneration will occur. The mystifying phenomenon of an isolated piece restoring its lost organs thus

turns out to be the result of two plain chemical factors: the law of mass action and the production and giving off of inhibitory substances in the growing regions of the organism.

¹ Loeb, J., *Science, New York*, **45**, 1917, (436); *Bot. Gaz., Chicago*, **65**, 1918 (150).

² Loeb, J., *Science, New York*, **46**, 1917, (547).

THE INFLUENCE OF NEUTRAL SALTS UPON THE VISCOSITY OF GELATIN SOLUTIONS.

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I.

When dried pig's bladder or powdered gelatin is treated with a neutral salt a certain swelling occurs; after the salt is washed away a much greater additional swelling is noticed when the cation of the neutral salt used was monovalent. When the cation of the salt used was bivalent no such additional swelling occurs.¹ These phenomena had been explained by the writer on the assumption that neutral salts with monovalent cation (Li, Na, K, NH₄) form with gelatin and certain other proteins highly ionizable metal proteinates which dissociate electrolytically into a positive metal ion (that of the salt used) and a negative protein (gelatin) ion. It is the latter which is responsible for the additional swelling after the excess of the salt is washed away, the ionization of the metal gelatinate being repressed in the presence of the excess of salt. The salts with bivalent cation (Mg, Ca, Sr, Ba) were assumed to form non- or less ionizable metal gelatinates and hence no or little additional swelling of gelatin was observed after the excess of neutral salts of bivalent cations was washed away.^{2, 3}

The new experiments reported in this note were undertaken with the intention of testing the idea that the difference in the influence of the monovalent and bivalent cations upon the additional swelling is due to a difference in the degree of ionization of the metal gelatinates with monovalent and bivalent cations.

¹ Loeb, J., *J. Biol. Chem.*, 1917, xxxi, 343.

² Loeb, J., *J. Biol. Chem.*, 1918, xxxiii, 531.

³ Loeb, J., *J. Biol. Chem.*, 1918, xxxiv, 77.

It has been pointed out by various authors—Laqueur and Sackur,⁴ Hardy,⁵ Pauli,⁶ Bottazzi,⁷ and Robertson⁸—that solutions of ionized protein have a much higher viscosity than solutions of the same protein in non-ionized condition. These statements are based on experiments with acids and bases, but not with neutral salts. If they are correct—as they seem to be—then the measurements of viscosity should offer a means of testing our hypothesis that neutral salts with univalent metal (type NaCl and Na₂SO₄) react with gelatin under formation of highly ionizable metal gelatinates (*e.g.* sodium gelatinate), while neutral salts with bivalent metal (type CaCl₂) form metal gelatinates (*e.g.* calcium gelatinate) which are not or only slightly capable of ionization.

II.

The method of our procedure was as follows. 1 gm. of finely powdered gelatin was put into 100 cc. of a neutral salt solution, whose concentration was generally M/8, and left in this solution for about 1 hour. The powder was stirred repeatedly in the solution. It was then poured into one of the cylindrical funnels described in the previous papers and the salt solution was allowed to filter off as much as possible. To remove the last traces of salt solution the gelatin mass was then perfused four times with 25 cc. of H₂O. Tests with silver nitrate showed that the water filtering off in the fourth perfusion no longer gave a precipitate when the salt used was a chloride. The removal of the salt solution is necessary since its presence represses the ionization of the metal gelatinate formed. It was the neglect of this precaution which led to the erroneous results of some previous experimenters on the action of neutral salts on proteins. Hence our viscosity measurements were all made with gelatin freed from the excess of the salt (with which it had been

⁴ Laqueur, E., and Sackur, O., *Beitr. chem. Physiol. u. Path.*, 1903, iii, 193.

⁵ Hardy, W. B., *J. Physiol.*, 1905–06, xxxiii, 251.

⁶ Pauli, W., *Fortschr. naturwiss. Forschung*, 1912, iv, 223.

⁷ Bottazzi, F., *Atti. Accad. Lincei*, 1913, xxii, pt. 2, 141, 263.

⁸ Robertson, T. B., *The physical chemistry of the proteins*, New York, 1918, 320.

treated) and dissolved in distilled water (and not in the salt solution with which the gelatin had previously been treated).

It is well known that the viscosity of gelatin solution increases with standing even if the solution remains liquid. This has been ascribed to the fact that the gelatin solution has a similar structure to the solid gelatin,⁸ but that this structure is only gradually reached by the liquid. We may say that gelatin solution upon standing changes towards a gel structure though it may never reach the actual gel stage. Since we now know through the work of Langmuir that molecules are not spherical, but that we may have to conceive them in the case of proteins as having a chain structure, we may assume that the steady increase of viscosity of gelatin solution upon standing is due to a gradual orientation of gelatin molecules or ions towards the arrangement they possess in a solid gel. On that assumption we understand also the influence of temperature upon the viscosity of gelatin solutions.

Reproducible measurements of viscosity of gelatin solutions can only be obtained when these two chief influences, time and temperature, are kept equal. Our mode of procedure was as follows.

After the mass of gelatin had been washed free from the salt solution with which it had been treated it was liquefied by heating in a water bath at about 50°C. for about 10 minutes. The solution containing 1 gm. of the dry gelatin was then made up to a volume of 100 cc. by adding the necessary amount of *distilled water* and the temperature of the solution lowered to 24°C. The viscosity was determined *immediately* at that temperature, the Ostwald viscometer being in a water bath of 24°C.

In this way we got comparatively constant and reproducible results. The slight variations observed for the same solution were due to the fact that in some cases the gelatin was liquefied immediately after perfusion while in other experiments we waited until the next morning. In the latter case the viscosity was always lower, possibly on account of some hydrolysis or putrefaction.

The time of outflow of distilled water through our viscometer was 53 seconds at 24°C.

A 1 per cent gelatin solution made up of powdered gelatin washed repeatedly with distilled water gave in different experiments the fol-

lowing times of outflow: 84, 83, 78, 84, 86, 80, 83, 76, 83, 82, 83, and 84 seconds, averaging 82 seconds. We may therefore say, a 1 per cent solution of the gelatin used by us (possessing a negligible degree of electrolytic dissociation) has a viscosity corresponding to a time of outflow of 82 seconds through our viscometer.

It seemed advisable to determine the influence of the concentration of the salt solution used upon the result. For this purpose 1 gm. of finely powdered Cooper's non-bleached gelatin was put for 1 hour into each of a series of beakers containing 100 cc. of a NaCl solution varying from $M/2$ to $M/1024$ in concentration. After this the gelatin from each solution was put into a separate cylindrical funnel, drained off, and perfused four times with distilled water.⁹ The water running off in the fourth perfusion was practically free from chloride when the gelatin had been treated with an $M/8$ solution of NaCl. The gelatin was therefore free from salt except that held in chemical combination. Three effects of the neutral salts upon the gelatin were measured, namely:

1. The additional swelling; *i.e.*, the increase in swelling which occurred in the cylindrical column of gelatin after the salt was washed away (and the ionization was no longer repressed). The additional swelling was measured in mm. of the height of the column of gelatin in the cylindrical funnels which all had the same diameter.

2. The viscosity of the washed gelatin. The viscosity was expected to increase parallel with the additional swelling, if both additional swelling and increase in viscosity were due to the same factor; namely, increased ionization of the gelatin. The viscosity measurements are given in times (seconds) of outflow through the viscometer.

3. The amount of 95 per cent alcohol required to precipitate 5 cc. of a 1 per cent gelatin solution (washed) and made up in distilled water. According to Pauli, ionized protein loses its precipitability by alcohol. Hence when the ionization of the gelatin exceeds a certain limit it should cease to be precipitable by alcohol. We assumed that this limit was reached when 25 cc. or more of alcohol no longer gave a precipitate and we indicate this by the symbol ∞ .

⁹ Those treated with $M/2$ and $M/4$ NaCl were perfused six times with 25 cc. of distilled water.

The reader will notice that in all cases the measurements were made on gelatin in the *absence* of the salt solution with which it had been treated. As has been stated in this and the previous paper, the measurements in the presence of salts are of restricted value, since the ionizing effect of the salt on the protein is concealed in the presence of the salt.

Table I gives the three groups of measurements.

TABLE I.

1 gm. gelatin treated for 1 hour with one of the following solutions of NaCl and then freed from the supernatant solution by four perfusions with 25 cc. of distilled water.											
	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	H ₂ O (control).
Additional swelling in mm. of the height of the cylindrical column of gelatin.....	43	40	41	38	40	30	24	20	9	9	5.5
Viscosity of 1 per cent gelatin solution in H ₂ O in seconds of time of outflow.....	99	99	98	97	95	93	90	89	87	86	85
Cc. of 95 per cent alcohol required to precipitate 5 cc. 1 per cent gelatin solution in H ₂ O.....	∞	∞	∞	∞	∞	∞	11.3	6.6	5.7	5.7	5.5

These measurements show that beyond a concentration of M/8 a further increase of concentration does not influence the results materially. We therefore used salts with univalent ions in this concentration, while salts with bivalent ions were generally though not always used in M/16 concentration.

III.

We now proceeded to test our hypotheses, namely:

1. That neutral salts with univalent cation (type NaCl and Na₂SO₄) form with common gelatin highly ionizable gelatin compounds (metal gelatinates).

2. That neutral salts of the alkali earth metals (type CaCl₂) form compounds with gelatin (metal gelatinates) which are only slightly dissociable.

If these hypotheses are correct, we should expect, first, that gelatin treated for 1 hour with an $M/8$ solution of any neutral salt with univalent cation (Li, Na, K, NH_4) should (when freed from the excess of salt by perfusion with H_2O) have a considerably increased viscosity; *i.e.*, a time of outflow considerably higher than 82 seconds. Moreover, the time of outflow should be exactly the same for all the salts used regardless of the nature of the cation as long as this is univalent, and regardless of the nature of the anion, whether Cl, NO_3 , acetate, sulfate, tartrate, succinate, etc.; the only provision being that the salt solution originally applied should always have the same concentration of the univalent cation ($M/8$ in our experiments). All this turned out as our theory demanded. In our experiments 1 gm. of powdered gelatin was allowed to interact for 1 hour with an $M/8$ solution of salts of the type NaCl and with $M/16$ solutions of salts of the type Na_2SO_4 (bivalent anion). When the salt in the capillary spaces of the gelatin granules was washed away with distilled water, the times of outflow were found to be about 97 seconds (varying between 95 and 102), regardless of the nature of the anion or cation (as long as the latter was univalent) of the salt used. This was an increase in the viscosity of about 20 per cent.

Second, if our hypothesis is correct, gelatin treated for 1 hour with $M/8$ or $M/16$ solutions of neutral salts of the alkaline earth metals should, when the salt solution in the capillary spaces of the powdered mass is washed away with H_2O , give times of outflow not much higher than those found for common gelatin (82 seconds). This also was found to be the case. Gelatin treated previously for 1 hour with $M/8$ or $M/16$ $MgCl_2$, $CaCl_2$, $SrCl_2$, $BaCl_2$, gave outflows between 82 and 87, which is only a slight if any increase over that of common gelatin.

Gelatin treated with $M/8$ cane sugar, $M/8$ glycerol, or $M/8$ ethyl alcohol, gave times of outflow identical with those found for common gelatin. Table II gives the full results. The slight variations between members of the same groups of salts are due to the fact that some viscosity determinations were made on the same day on which the gelatin was treated with the salt solution and others on the day following.

TABLE II.

Type of salt.	Molecular concentration of salt.	Nature of salt with which gelatin was treated.	Viscosity (expressed in time of outflow) of 1 per cent gelatin solution; gelatin treated with salt, then with H ₂ O to wash away salt.
			<i>sec.</i>
I. Univalent cation, univalent anion.	M/8	NaCl.....	95
	M/8	NaNO ₃	97
	M/8	Na acetate.....	95
	M/8	NaCNS.....	96
	M/8	KCl.....	99
	M/8	LiNO ₃	96
	M/8	KCNS.....	96
	M/8	LiBr.....	99
	M/8	NH ₄ Br.....	96
II. Univalent cation, bivalent anion.	M/8	Na ₂ SO ₄	95
	M/8	Na ₂ oxalate.....	100
	M/16	Na ₂ tartrate.....	99
	M/16	Na ₂ malate.....	97
	M/16	Na ₂ succinate.....	100
	M/16	Li ₂ SO ₄	99
	M/16	K ₂ SO ₄	99
	M/16	(NH ₄) ₂ SO ₄	98
	M/16	K ₂ tartrate.....	102
III. Bivalent cation, univalent anion.	M/8	MgCl ₂	87
	M/16	CaCl ₂	82
	M/8	SrCl ₂	87
	M/16	BaCl ₂	82
IV. Bivalent cation, bivalent anion.	M/8	MgSO ₄	90
V. Non-electrolytes.		H ₂ O.....	82
	M/8	Glycerol.....	79
	M/8	Cane sugar.....	80
	M/8	Ethyl alcohol.....	81

If it is true that the increase in viscosity is the expression of an increase in ionization of the gelatin, the facts found by us support our hypotheses: (1) that neutral salts with univalent cations form with gelatin highly ionizable metal gelatin^{ate} compounds which

dissociate into a positive metal and a negative gelatin ion; and (2) that neutral salts with bivalent cation form metal gelatinates which are considerably less ionizable. As far as the anion is concerned, its influence is neither noticeable in the additional swelling nor in the increase in viscosity. MgSO_2 and CaSO_2 act not differently from MgCl_2 and CaCl_2 , and NaCl not differently from Na_2SO_4 , as long as the concentration of the cation is the same. This means that the anion of the salt used either does not combine with the protein at all or that if it combines it enters into a bondage where its influence is not noticeable.

Finally, the results on viscosity support our statement drawn from experiments on swelling that the action of neutral salts upon gelatin is of a stoichiometrical character, since the increase in the viscosity of the gelatin caused by a previous treatment with a salt solution is exactly the same for all salts with univalent cations regardless of the nature of the anion or cation as long as the concentration of the cation is the same. This stoichiometrical result is entirely independent of the hypothesis that the increase in viscosity is due to an increase in ionization.

IV.

If we treat gelatin with HCl , gelatin chloride (or hydrochloride) is formed, which according to our previous observations should react only with the anion of neutral salts, since such a compound dissociates preeminently or exclusively into a positive gelatin ion and a negative chlorion. Hence we should expect that a treatment of gelatin chloride with salts of the calcium group would affect the viscosity of gelatin chloride (after the salt solution is washed away) qualitatively and quantitatively like the treatment with salts of the type NaCl , provided the concentration of the Cl ion is the same; while the influence of the cations, Na and Ca , which was so strong in the case of common gelatin, should not be noticeable. All this turned out as expected. Hence neutral salts of the calcium group as well as neutral salts of the sodium group form highly ionizable salts with gelatin chloride—as long as the anion of the salt is univalent. Neutral salts with bivalent anion, however, should form with gelatin chloride new compounds (gelatin sulfate, gelatin oxalate, gelatin

malate, etc.) which are less ionizable than gelatin chloride (or hydrochloride), and this was also borne out by the experiments which we will now describe in more detail.

1 gm. of powdered gelatin was put for half an hour into a beaker, containing 100 cc. $M/100$ HCl and the gelatin was repeatedly stirred. The gelatin was then put into the cylindrical funnel and the HCl solution (or what was left of it) allowed to filter off. After this the gelatin in the funnel was perfused three times with 25 cc. of the salt solution ($M/8$ or $M/16$ respectively) and then four times with 25 cc. of H_2O to remove any free salt solution. After this water had run off the gelatin was made up into a 1 per cent solution (in distilled water) and its viscosity was determined immediately as described.

The time of outflow of liquid gelatin chloride produced by treatment with $M/100$ HCl, when perfused three or four times with water to remove the remnant of acid in the capillary spaces between the gelatin granules, was for a 1 per cent solution generally between 120 and 130 seconds, and sometimes higher.

Gelatin chloride produced by a half hour treatment of 1 gm. of powdered gelatin in 100 cc. $M/100$ HCl was put into a funnel, the HCl solution was allowed to filter off, and the gelatin was perfused three times with $M/8$ $MgCl_2$ or $CaCl_2$ or $SrCl_2$, and then perfused four times with H_2O . The time of outflow varied between 132 and 135 seconds (Table III), a value which was identical with the one obtained when salts with a monovalent cation and anion were used for perfusion, such as NaCl, $NaNO_3$, KCl, KCNS, LiBr, NH_4Br , etc., the time of outflow varying in this case between 129 and 136 seconds (Table III). Only Na acetate proved to be an exception as was the case in similar experiments on swelling. This suggests that in the reaction between gelatin chloride and neutral salts only the anion of the neutral salts reacts with the gelatin or that at least the effect of the cation is not noticeable. This is exactly the same conclusion to which our experiments on swelling had led us. On the other hand, the same theory demanded that when gelatin chloride reacts with salts with bivalent anion, like SO_4 , oxalate, etc., gelatin sulfate, gelatin oxalate, etc., should be formed with a much lower degree of ionization and hence a much lower degree of viscosity.

This was also found to be true. When gelatin chloride obtained by interaction for half an hour between 100 cc. M/100 HCl and 1 gm. gelatin was perfused three times with one of these salts with bivalent anion and then freed from the salt by washing four times with

TABLE III.

Type of salt.	Molecular concentration of salt.	Nature of salt with which gelatin was treated.	Viscosity (expressed in time of outflow) of 1 per cent gelatin solution; gelatin first treated with M/100 HCl, then with salt, then with H ₂ O to wash away salt.
			<i>sec.</i>
I. Univalent cation, univalent anion.	M/8	NaCl.....	131
	M/8	NaNO ₃	136
	M/8	Na acetate.....	107
	M/8	KCl.....	131
	M/8	LiNO ₃	130
	M/8	KCNS.....	129
	M/8	LiBr.....	132
	M/8	NH ₄ Br.....	132
II. Univalent cation, bivalent anion.	M/8	Na ₂ SO ₄	105
	M/8	Na ₂ oxalate.....	94
	M/16	Na ₂ tartrate.....	102
	M/16	Na ₂ malate.....	109
	M/16	Na ₂ succinate.....	106
	M/16	(NH ₄) ₂ SO ₄	106
	M/16	K ₂ tartrate.....	97
III. Bivalent cation, univalent anion.	M/8	MgCl ₂	132
	M/8	CaCl ₂	133
	M/8	SrCl ₂	135
IV. Bivalent cation, bivalent anion.	M/8	MgSO ₄	103

H₂O, the viscosity was lowered more than 20 per cent, the time of outflow varying between 94 and 109 seconds (Table III). Hence sulfates, oxalates, malates, succinates, act upon gelatin chloride in the same way as do Ca or Mg or Ba upon common gelatin; namely, forming little or less dissociable gelatin salts. This agrees with the

conclusion drawn from the experiments on the effect of neutral salts upon the swelling of gelatin.

The fact that gelatin treated with $M/100$ HCl has a higher viscosity (and swells more) than gelatin treated with $M/8$ NaCl may be due to the difference in the mass of gelatin salt formed in the two cases.

V.

A third test for our theory should consist in the proof that the influence of neutral salts upon the viscosity of sodium gelatinate produced by the action of NaOH upon 1 gm. of gelatin runs parallel to the influence of neutral salts upon the viscosity of common gelatin. The test turned out as our theory demanded.

1 gm. of finely powdered gelatin was put for half an hour into a beaker containing 100 cc. $M/100$ NaOH. The gelatin was then put into a funnel in order to allow the NaOH to filter off, and after this the gelatin was perfused three times with 25 cc. of an $M/8$ or $M/16$ salt solution and four times with 25 cc. of H_2O to wash out the salt solution from the capillary spaces. The results were as follows.

When gelatin is treated with $M/100$ NaOH the viscosity measured in times of outflow is raised from 82 seconds to from 115 to 118 seconds. When it is washed with water a slightly lower value is obtained, due possibly to the washing away of some of the NaOH formed by hydrolytic dissociation of sodium gelatinate with subsequent diminution of sodium gelatinate. The value 115 to 118 seconds is higher than the value obtained by the interaction between $M/8$ NaCl and gelatin, which was about 98 seconds, indicating that more sodium gelatinate is formed by the interaction of 1 gm. of gelatin with 100 cc. $M/100$ NaOH than with 100 cc. $M/8$ NaCl.

The theory which we developed on the basis of the swelling experiments demands that if sodium gelatinate produced by the action of $M/100$ NaOH be treated with a neutral salt with univalent cation the viscosity should be the same no matter what the nature of the anion or whether the latter is monovalent or bivalent, provided that the concentration of the cation is the same in all cases and provided that the salt in the capillary spaces between the granules of gelatin is washed away before the viscosity is determined. The experiments

confirmed this expectation. For salts of the type NaCl as well as for salts of the type Na_2SO_4 , the viscosity measured in time of outflow was the same; namely, in the neighborhood of 118 (Table IV). This again indicates that only the cation of the neutral salt interacts

TABLE IV.

Type of salt.	Molecular concentration of salt.	Nature of salt with which gelatin was treated.	Viscosity (expressed in time of outflow) of 1 per cent gelatin solution; gelatin first treated with $\text{m}/100$ NaOH, then with salt, then with H_2O to wash away salt.
			<i>sec.</i>
I. Univalent cation, univalent anion.	$\text{m}/8$	NaCl.....	115
	$\text{m}/8$	NaNO_3	119
	$\text{m}/8$	Na acetate.....	120
	$\text{m}/8$	KCl.....	120
	$\text{m}/8$	LiNO_3	116
	$\text{m}/8$	KCNS.....	116
	$\text{m}/8$	LiBr.....	118
	$\text{m}/8$	NH_4Br	110
II. Univalent cation, bivalent anion.	$\text{m}/16$	Na_2SO_4	116
	$\text{m}/16$	Na_2 oxalate.....	112
	$\text{m}/16$	Na_2 tartrate.....	120
	$\text{m}/16$	Na_2 malate.....	120
	$\text{m}/16$	Na_2 succinate.....	119
	$\text{m}/8$	$(\text{NH}_4)_2\text{SO}_4$	110
	$\text{m}/16$	K_2 tartrate.....	117
III. Bivalent cation, univalent anion.	$\text{m}/16$	MgCl_2	87
	$\text{m}/16$	CaCl_2	96
	$\text{m}/8$	SrCl_2	98
IV. Bivalent cation, bivalent anion.	$\text{m}/8$	MgSO_4	103

with sodium gelatinate. When MgCl_2 , CaCl_2 , and SrCl_2 were substituted for the salts with univalent cation the time of outflow was reduced to from 87 to 98 seconds (Table IV), indicating that lowering of the degree of electrolytic dissociation which our theory demanded. This lowering was due to the formation of non- or less

dissociable calcium gelatinate or magnesium gelatinate from sodium gelatinate.

VI.

Attention had been called in this and the writer's former papers to the fact that the additional swelling due to the action of a neutral salt upon gelatin is only noticeable after the excess of salt solution has been washed away, and this was explained on the assumption that the additional swelling was due to the gelatin ions formed under the influence of the salt, but owing to the repression of ionization in the presence of the salt the additional swelling could only show itself after the excess of salt solution was washed away. The same is true for the influence of salts upon the viscosity. When we measure the influence of neutral salts upon the viscosity of gelatin solution in the presence of the salt we get negative if not confusing results. Thus measurements of the viscosity of gelatin in $M/8$ $MgCl_2$, $CaCl_2$, or $SrCl_2$ gave times of outflow between 74 and 85 seconds, while the viscosity measurements of gelatin in the presence of salts of the type $NaCl$ gave values between 72 and 76 seconds, and in the presence of salts of the type Na_2SO_4 the times were 80 to 83 seconds. Almost similar figures were obtained for gelatin treated first with $M/100$ HCl and then with $M/8$ neutral salts. This agrees with our theory that the effect of neutral salts upon the additional swelling and upon the increase of viscosity is due to the ionization of gelatin salts with univalent anion or cation; and that this ionization is repressed by the presence of as high a concentration of a neutral salt as $M/8$ or $M/16$. This confirms our statement that any theory of the action of the neutral salts upon proteins must be based on experiments in which the excess of salts which have acted on the protein has been washed away.

VII.

We have called attention to the fact that if dried pig's bladder or powdered gelatin is treated with 100 cc. $M/8$ $NaCl$ containing quantities of $M/8$ $CaCl_2$ varying from 1 to 32 cc. the additional swelling noticeable after the salt is washed away diminishes with the amount of $CaCl_2$ added. This is a case of antagonistic salt ac-

tion. It was of interest to find out whether this antagonistic effect of the addition of CaCl_2 to NaCl would also become noticeable in the viscosity of the gelatin after the salt was washed away. This was the case. 1 gm. of powdered gelatin was put for 1 hour into 100 cc. $\text{M}/8$ NaCl containing 0, 1, 2, 4, 8, 12, 16, and 32 cc. $\text{M}/8$ CaCl_2 . As controls gelatin treated with H_2O alone and gelatin treated for 1 hour with $\text{M}/8$ CaCl_2 were added. After staying 1 hour in these solutions the gelatin was put into the cylindrical funnels where it was washed four times with 25 cc. of distilled water, until the filtrate no longer gave a precipitate with silver nitrate. The additional swelling (expressed in mm. of the height of the cylindrical column of gelatin), the viscosity expressed in time of outflow, and the alcohol precipitability of 1 per cent gelatin solutions treated in the way mentioned are given in Table V. The reader's attention is called to the fact that all the measurements were made on gelatin washed free from the salt originally used and dissolved in H_2O .

TABLE V.

1 gm. gelatin put for 1 hour into one of the following solutions and then put into funnels and perfused four times with distilled water.									
	100 cc. $\text{M}/8$ NaCl + cc. $\text{M}/8$ CaCl_2 .							$\text{M}/8$ CaCl_2	H_2O
	0	1	2	4	8	16	32		
Additional swelling in mm. of the cylindrical column of gelatin.....	36	38	35	25	23	22	17	6.0	6.5
Viscosity of 1 per cent gelatin solution in H_2O in seconds of time of outflow.....	98	96	96	92	91	88	90	88	84
Cc. of 95 per cent alcohol required to precipitate 5 cc. 1 per cent gelatin solution in H_2O	∞	∞	∞	∞	About 20	9.5	6.5	5.4	5.5

The additional swelling is markedly lowered by the addition of 4 cc. or more of $\text{M}/8$ CaCl_2 to 100 cc. $\text{M}/8$ NaCl , and the addition of 1 cc. or more of CaCl_2 lowers the viscosity. The precipitability with alcohol begins when more than 8 cc. $\text{M}/8$ CaCl_2 are added to 100 cc. $\text{M}/8$ NaCl .

Viscosity and additional swelling give parallel results, supporting our view that in this case the antagonism is due to the diminution in the ionization of a metal gelatin through the transformation of the highly ionizable sodium gelatinate into calcium gelatinate which is only slightly ionizable.

There exists a second type of antagonistic salt action due to the transformation of the highly ionizable gelatin chloride into the less ionizable gelatin sulfate. It was of interest to apply the three tests, additional swelling, viscosity measurements, and precipitation with alcohol, to this assumption.

1 gm. of powdered gelatin was put for half an hour into each of a series of beakers containing 100 cc. M/100 HCl to transform the gelatin into gelatin chloride or hydrochloride. The gelatin was then put into a series of cylindrical funnels which were perfused three times with 25 cc. of an M/8 solution of NaCl containing varying quantities of M/16 Na₂SO₄; namely, the following mixtures, 100 cc. M/8 NaCl containing 0, 1, 2, 4, 8, 16, and 32 cc. M/16 Na₂SO₄. As a control one funnel was perfused with M/16 Na₂SO₄. After this each funnel was perfused four times with 25 cc. H₂O to wash away the salt solution in the capillary spaces between the granules.

Table VI gives the results. The additional swelling is the swelling observed at the end of the washing with water, in excess of the swelling which occurred in the gelatin while the latter was in the acid solution. The reader will notice that the addition of Na₂SO₄ to NaCl diminishes the ionizing effect of the NaCl, inasmuch as with the increase in the quantity of Na₂SO₄ added to the NaCl solution the additional swelling, as well as the viscosity, is diminished (Table VI).

It is obvious again that the values for viscosity are extremely regular and that they diminish with the amount of Na₂SO₄ added. The viscosity of a 1 per cent gelatin solution only perfused with H₂O corresponded to a time of outflow of 86 seconds.

These viscosity measurements therefore support our hypothesis that antagonistic salt action can be produced by the transformation of a more ionizable gelatin salt of the type sodium gelatinate into a less ionizable gelatin salt of the type calcium gelatinate; or of the more ionizable salt of the type gelatin chloride into a less ionizable salt of the type gelatin sulfate.

TABLE VI.

1 gm. gelatin put for 30 min. into 100 cc. $M/100$ HCl to produce gelatin chloride and then put into funnels and perfused three times with 25 cc. of the following salt solutions and four times with 25 cc. H_2O to wash away the salt.

	100 cc. $M/8$ NaCl + cc. $M/16$ Na_2SO_4 .							$M/16$ Na_2SO_4
	0	1	2	4	8	16	32	
Additional swelling in mm. of the height of the cylindrical column of gelatin.....	36	34	29	25.5	26.5	24	14	-5.5
Viscosity of 1 per cent gelatin solution in H_2O in seconds of time of outflow..	138	137	135	131	130	125	118	108
Cc. of 95 per cent alcohol required to precipitate 5 cc. 1 per cent gelatin solution in H_2O	∞	∞	∞	∞	∞	∞	∞	9

Theoretical Remarks.

In three former papers we have shown that when powdered gelatin or dried pig's bladder is treated with the solution of a neutral salt, the gelatin or bladder undergoes a certain amount of swelling and that after the salt solution is washed away with distilled water a considerable additional swelling of the gelatin occurs. In the same papers we had also shown that the influence of neutral salts upon the additional swelling of gelatin or pig's bladder is of a stoichiometrical character, depending purely upon the concentration and valency of the cation of the salt used. Our present experiments on the influence of neutral salts upon the viscosity of gelatin solution show that the influence of neutral salts upon the viscosity is also of a stoichiometrical character, inasmuch as it also depends exclusively or preeminently upon the concentration and valency of the cation of the neutral salt used. The anion seemed to be without influence. The writer had found in his former experiments that the neutral salts with monovalent cation cause a considerable additional swelling of the gelatin while the neutral salts with bivalent cation cause no such swelling, and to explain this difference he had assumed that neutral salts with monovalent cation form with gelatin metal gelatinates which are highly ionizable, while salts with bivalent cations form

with gelatin metal gelatinates which are much less or only slightly ionizable. The experiments on viscosity support this conclusion, if the statement generally made is correct that ionization increases the viscosity of a protein solution.

In publications on the peculiarities of colloids the statement is frequently made that the oppositely charged ions of a neutral salt affect a colloid, *e.g.* a protein, in the opposite sense and that the effect of the salt is always the algebraic sum of these two opposing forces. This statement does not hold for the influence of neutral salt on those qualities of colloids which have thus far formed the subject of our investigation; namely, additional swelling, viscosity, and precipitability by alcohol after the salt has been washed away. Common gelatin (and the same may be true for all proteins which are much stronger as acids than as bases) reacts with neutral salts as if only the cation of the salt were active; salts of the type NaCl and of the type Na_2SO_4 act qualitatively and quantitatively alike as long as the concentration of the cation is the same, and the nature of the anion does not influence the result. It was also possible to show that MgCl_2 acts like MgSO_4 and CaCl_2 like CaSO_4 as long as the concentration of the cation is the same. All this would be impossible if the anion acted upon the protein in a sense opposite to that of the cation. The qualities which we have investigated depend apparently on the ionized negative gelatin ion and this combines only with the cation of the neutral salt applied. If the anion of the latter enters at all into combination with the negative gelatin ion its influence does not show itself in those qualities which thus far have formed the subject of our investigations.

When we produce gelatin chloride (or hydrochloride) by treating the gelatin with $\text{m}/100$ HCl , and wash away the superfluous acid the gelatin chloride is only influenced by the anion of a neutral salt, and salts of the type CaCl_2 act qualitatively and quantitatively like salts of the type NaCl if the concentration of the Cl ion is the same. The cation does not act in the opposite sense from the anion of the salt but does not act at all, or at least its influence is not noticeable, and Ca , which influences common gelatin and sodium gelatinates produced by treatment with NaOH so powerfully, is apparently without influence when acting upon gelatin chloride. Our experiments have

therefore led to a remarkable simplification of our conception of the influence of neutral salts upon proteins inasmuch as they show that for the physical characters thus far studied by us only one of the ions of the neutral salts is to be considered; namely, the one which has the opposite charge from the protein ion with which it reacts.

There are other qualities of proteins which are not confined to the ionized protein. Our investigations have not yet been extended to these qualities and we are not prepared to say whether or not qualities depending upon the non-dissociated protein molecules are influenced in an opposite sense by the oppositely charged ions of neutral salts.

SUMMARY.

1. Our experiments have shown that when we treat gelatin with $M/8$ or $M/16$ solutions of neutral salts with univalent cation, the viscosity of the gelatin is increased considerably after the excess of salt is washed away. When we treat gelatin with $M/8$ or $M/16$ solutions of neutral salts with bivalent cation no or only a slight increase of viscosity of the gelatin is noticeable after the excess of salt is washed away.

2. Neutral salts with univalent cation (Li, Na, K, NH_4) produce the same increase in the viscosity of the gelatin solution as long as the concentration of cation is the same,—namely $M/8$ in our experiments,—regardless of the nature of the anion and regardless of whether the anion is univalent or bivalent.

3. The statements made concerning the influence of neutral salts upon the viscosity of gelatin hold also for the influence of these salts upon the viscosity of gelatin previously treated with an alkali; *e.g.*, NaOH.

4. The action of neutral salts upon the viscosity of gelatin previously treated with HCl shows that salts with bivalent anion lower the viscosity of such gelatin; while salts with univalent anion have the opposite effect. The influence of the cation was not noticeable in our experiments.

5. These observations do not agree with the statement frequently made that the action of a neutral salt upon a protein is the algebraic sum of the opposite action of the oppositely charged ions of the

neutral salts. The viscosity of a gelatin solution is influenced chiefly if not exclusively by one of the two ions of the neutral salt; namely, the one which has the opposite electrical charge from that of the protein. Since common gelatin is stronger as acid than as base, its viscosity is influenced only by the cation of a neutral salt; and the same is true for the influence of neutral salts upon the viscosity of metal gelatinates produced by the action of bases upon gelatin. The viscosity of gelatin-acid salts (produced by the action of acids upon gelatin) which yield a positive gelatin ion is influenced chiefly if not exclusively by the anion of a neutral salt.

6. These conclusions are in agreement with the conclusions drawn from the experiments on the limiting concentration of neutral salts upon additional swelling reported in the writer's previous publications.

7. If it is true that the increase in the viscosity of gelatin solution under the influence of electrolytes is due to an increase in the ionization of the gelatin, our results support the hypothesis that neutral salts with univalent cation form highly ionizable metal proteinates with proteins of the type of gelatin (which is much stronger as acid than as base), while neutral salts with bivalent cation form less or non-dissociable metal proteinates with gelatin.

THE INFLUENCE OF NEUTRAL SALTS, BASES, AND ACIDS ON THE PRECIPITABILITY OF GELATIN BY ALCOHOL.

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I.

It has been shown in the previous publications¹ that the effect of neutral salts on gelatin is repressed in the presence of the salt and that we can study such effects with any profit only when the excess of salt is washed away after it has had a chance to act on the protein. Such gelatin reveals qualities which are masked in the presence of the salt and which allows us to gain a clear insight into the effect of the salt. The first striking result was that contrary to the current belief that the cation of the salt was found to be the active ion, while the anion did apparently not act at all. All salts with univalent cation and bivalent anion (type Na_2SO_4 or K_2 oxalate) had exactly the same effect as all the salts of the type NaCl or NH_4Cl (univalent cation and univalent anion) when the cation concentration was the same in solutions of both salts. While the salts with univalent cation caused an additional swelling and an increase in viscosity of the gelatin after the salt was washed away, the salts with bivalent cation caused little or no such additional swelling and no or only a slight increase in viscosity.

This difference in the effect of univalent and bivalent cations, as well as the repressing effect of the presence of the salt, was explained on the assumption that the neutral salts form with gelatin metal gelatinates which dissociate electrolytically into a negative gelatin ion and a positive cation (that of the salt used), while the anion either does not combine at all with the gelatin or forms a non-

¹ Loeb, J., *J. Biol. Chem.*, 1917, xxxi, 343; 1918, xxxiii, 531; xxxiv, 77, 395.

or less dissociable compound. Univalent cations, like Na, form gelatinates which are highly dissociable electrolytically, while the bivalent metals form non- or less dissociable compounds. The presence of the salt represses the electrolytic dissociation of the metal proteinate formed and this repressing effect makes it necessary to remove the excess of salt if we wish to study the effect of the salt on the protein, since many of these effects are due to the ionizing action of the salt on the protein as a consequence of the formation of metal proteinates.

It was pointed out that these results lead to a considerable simplification of protein chemistry. According to our results, gelatin behaves like a weak acid, which, if in contact with a neutral salt, will exchange some of its H ions for the cations of the salt. The anions of the salt do not enter into combination with the gelatin or if they do their effect is not noticeable. When, however, we treat gelatin with a comparatively low concentration of a strong acid, *e.g.* $m/100$ HCl, gelatin chloride or hydrochloride is formed which should be able to exchange its anion when treated with a neutral salt, while the cation of the latter should not enter into any reaction with the gelatin, or if it does its effect should not be noticeable. This was found to be the case. NaNO_3 and $\text{Ca}(\text{NO}_3)_2$ act alike upon gelatin chloride when their NO_3 concentration is the same, and if the Ca and Na enter into combination with the gelatin chloride their influence is not noticeable. This contradicts the current statement, especially advocated by Pauli,² that the action of a neutral salt upon a protein is the algebraic sum of the opposite effects of the anion and cation of the salt used.

In this paper we wish to test our views further by investigating the effects of a treatment of gelatin by neutral salts upon the precipitability of the gelatin by alcohol. We have occasionally mentioned such investigations but have not yet published any systematic results.

The procedure was as follows. 1 gm. of Cooper's gelatin (grain size between sieve 50 and 60) was put into each of a series of beakers containing 100 cc. of various concentrations of a neutral salt, usually

² Pauli, W., *Beitr. chem. Physiol. u. Path.*, 1903, iii, 225.

from $M/4$ or $M/2$ down to $M/2048$.³ One beaker with distilled water served as a control. The gelatin powder remained in the beaker for 1 hour and was repeatedly stirred during this time. After 60 minutes the gelatin was poured into a cylindrical funnel and the salt solution was allowed to drain off. Then each cylinder was perfused six times with 25 cc. of H_2O to wash off all the salt solution contained in the capillary spaces between the granules.⁴ In the washing the additional swelling appeared which has been discussed in the previous papers. The mass of gelatin was then liquefied by heating for about 10 minutes in a water bath of $50^\circ C.$, and the amount of 95 per cent alcohol was ascertained which was required to precipitate 5 cc. of a 1 per cent solution of such gelatin at about $20^\circ C.$ The method here described has to be followed minutely if the same results as are described in this paper are to be obtained. Differences in the temperature and time of exposure of the gelatin to the salt solution, as well as differences in the amount of washing with distilled water, influence the results.

It is of interest to compare the quantity of alcohol required for precipitation of washed and non-washed gelatin (Table I). The sign ∞ means that the gelatin can no longer be precipitated or that no end-point can be reached with the addition of 20 cc. or more of alcohol.

The reader will notice at a glance the striking difference between the alcohol precipitability of gelatin in the presence of the salt and after the salt is washed away. In the latter case we have a striking *critical point*, which is identical for $NaCl$ and $LiCl$, namely at $M/128$, where the gelatin suddenly becomes non-precipitable, while at the next lower concentration ($M/256$) comparatively little alcohol was required for precipitation.

No such critical point exists when we test the alcohol precipitability of the 1 per cent gelatin solution in the presence of the salt

³ If the concentration of the salt is too high it may dissolve some of the gelatin.

⁴ On account of the hydrolytic dissociation of gelatin salts an excessive washing will finally lead to the formation of pure gelatin again. The washing, therefore, must be restricted to a removal of the free solution of the original electrolyte existing in the capillary spaces between the granules.

with which it had been treated. The quantities of alcohol required rise slowly with the concentration of the salt, but the differences are too small to select definite values as standards of comparison for the effect of different salts on the gelatin.

Experiments with acids and alkalines have led Pauli and his pupils to the conclusion that when the gelatin is sufficiently ionized it becomes non-precipitable by alcohol.⁵ If we apply this to our experiments with neutral salts we may draw the conclusion that if gelatin is put for 1 hour into a solution of NaCl or LiCl of $M/128$ or above, it is ionized to such an extent that it ceases to be precipitable by alcohol, provided the excess of salt is washed away, but that if

TABLE I.

		Cc. 95 per cent alcohol required for precipitation of 5 cc. 1 per cent solution of gelatin (at 20°C.) previously treated for 1 hour with												
		M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	H ₂ O
In presence of salt.	NaCl	10.0	8.6	7.4	6.2	5.3	4.1	3.9	4.1	3.4	3.3	3.3	3.4	3.3
	LiCl	11.05	10.45	8.7	7.55	6.65	6.9	5.8	4.55	5.15	3.55	3.25		3.6
In absence of salt (washed six times with H ₂ O).	NaCl	∞	∞	∞	∞	∞	∞	∞	∞	6.6	5.8	5.45		5.4
	LiCl	∞	∞	∞	∞	∞	∞	∞	∞	7.6	6.5	6.8	5.6	5.35

the salt is not washed away the ionization of the sodium or lithium gelatinate is repressed on account of the common ion; hence the gelatin remains precipitable in the presence of the salt.

Table I indicates still another fact worth mentioning; namely, that if we perfuse our powdered gelatin six times with 25 cc. of distilled water after it has been kept for 1 hour in 100 cc. of H₂O, it requires more alcohol for precipitation, namely about 5.5 cc., than when we soak it merely for 1 hour in distilled water; in the latter case it can be already precipitated by about 3.5 cc. of alcohol. The difference is due to the traces of salt which the gelatin contains in the form of impurities and which are removed by sufficient per-

* ⁵ Pauli, *Fortschr. naturwiss. Forschung*, 1912, iv, 223.

fusion with distilled water after it has been kept for 1 hour in distilled water. These impurities repress the ionization of the gelatin.

The reader will notice that the washed gelatin serving as control has about the same alcohol number in all the experiments reported in this paper. This alcohol number of washed gelatin indicates that normally a certain amount of ionization of gelatin into a negative gelatin ion and a positive hydrogen ion exists since we shall see later that if we add acid to make the gelatin isoelectric a 1 per cent solution precipitates spontaneously on standing at a low temperature and little or no alcohol is required to bring the precipitation to the standard used in these experiments (see Table IV).

Fenn⁶ has recently published a series of careful investigations on the influence of electrolytes upon the precipitation of gelatin by alcohol, but all his experiments as well as those of his predecessors on the same effect were made on gelatin in the presence of an excess of electrolytes used. As long as the experiments are made with very dilute acids or alkalies no serious error results, but in the case of higher concentrations of electrolytes (and in the case of salts higher concentrations are required) this method must give ambiguous results since the ionization of the gelatin will be repressed by the presence of the electrolyte. In our experiments this excess of electrolytes was washed away after they had had a chance to act on the gelatin and our experiments have led to entirely different results (Table II).

The reader will see from Table II that under our method of procedure all salts with univalent cation and univalent anion (type NaCl) render the gelatin solution non-precipitable in concentration of $M/128$ or slightly above (after the salt is washed away). For the majority of cases the critical concentration was $M/128$.

All salts with univalent cation and bivalent anion (type Na_2SO_4) make the gelatin solution non-precipitable with alcohol at exactly half the molecular concentration of salts of the type NaCl; namely, $M/256$ or slightly above. For all with the exception of one salt the value was $M/256$. This proves that the effect of the salt is determined exclusively by the cation and that the anion does apparently not influence the effect.

⁶ Fenn, W. O., *J. Biol. Chem.*, 1918, xxxiii, 279, 439; xxxiv, 141, 415.

When gelatin is treated with neutral salts of the alkali earth metals the gelatin remains precipitable with alcohol after the salt

TABLE II.

Type of salt.	Nature of salt.	Cc. 95 per cent alcohol required for precipitation of 5 cc. 1 per cent solution of gelatin (at about 20°C.) previously treated for 1 hour with 100 cc. of one of the following solutions and then freed from the excess of salt by perfusing six times with 25 cc. H ₂ O.												
		M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	H ₂ O
I. Univalent cation, univalent anion.	NaCl.....	8	8	8	8	8	8	8	8	6.6	5.8	5.45		5.4
	KCNS.....				8	8	8	8	8	6.8	6.0	5.6		5.5
	LiBr.....	8	8	8	8	8	8	8	8	7.3	6.2	6.0		5.7
	NH ₄ Cl.....	8	8	8	8	8	8	8	8	9.6	6.0	5.5	5.5	5.35
	LiCl.....	8	8	8	8	8	8	8	8	7.6	6.5	6.8	5.6	5.35
	KCl.....			8	8	8	8	8	8.6	6.2	4.8	5.1		5.0
	LiNO ₃			8	8	8	8	8	9.8	6.2	5.8	5.4		5.45
	Na acetate....	8	8	8	8	8	8	8	12.4	7.15	5.8	5.7	5.4	5.6
II. Univalent cation, bivalent anion.	Na ₂ SO ₄		8	8	8	8	8	8	8	∞	7.5	5.5		5.5
	Na ₂ oxalate...				8	8	8	8	8	∞	∞	6.8	6.3	5.85
	Na ₂ tartrate...	8	8	8	8	8	8	8	8	∞	8.0	6.6		5.7
	Na ₂ succinate..	8	8	8	8	8	8	8	8	∞	8.1	6.15	5.8	5.5
	K ₂ SO ₄			8	8	8	8	8	8	∞	6.85	6.0	5.7	5.45
	Li ₂ SO ₄			8	8	8	8	8	8	8.0	6.6	5.3		4.8
III. Bivalent cation, univalent anion.	MgCl ₂	6.2	6.3	7.05	7.25	7.6	7.4	7.4	6.2	6.7	5.3	5.8	5.6	5.4
	CaCl ₂			5.1	4.9	5.0	5.3	5.0	5.1	5.3	5.3	5.4	5.4	5.4
	SrCl ₂		5.3	4.95	5.2	5.6	6.0	5.8	5.5	5.5	5.5	5.5	5.4	5.4
	BaCl ₂			4.45	4.8	5.6	4.1	4.7	4.6	4.3	5.0	5.2	6.0	5.6
IV. Bivalent cation, bivalent anion.	MgSO ₄		6.3	6.5	6.3	6.4	6.6	6.4	6.4	5.5	5.5	5.1	5.1	5.1

is washed away no matter in what concentration the salt had been used.

A study of the alcohol numbers of the gelatin first treated with the salts of alkali earth metals and then freed from the excess of salt by washing with distilled water shows that only after the treat-

ment with MgCl_2 and MgSO_4 does the alcohol number rise slightly beyond that of gelatin washed with water but not previously treated with salt (under H_2O in Table II). Hence Mg is the only metal of this group which leads to a slight increase in ionization. It is also worth noticing that MgCl_2 and MgSO_4 act quantitatively alike, thus supporting our statement that the influence of the anion of the neutral salt does not make itself felt in these experiments.

These experiments on alcohol precipitation thus confirm the conclusion, drawn from our experiments on swelling and viscosity, that only the cation of the neutral salts influences the gelatin and that there is a typical difference in the action of univalent and bivalent cations.

The results can be best explained on the assumption that the neutral salts form with gelatin (and perhaps with all proteins which are stronger as acid than as base) metal proteinates, which are highly ionizable when the metal is univalent (Na , Li , K , NH_4) and much less ionizable when the salt used is bivalent (Mg , Ca , Sr , Ba).

II.

If this assumption is correct it follows that treatment of gelatin with solutions of NaOH or KOH should act exactly like treatment with solutions of neutral salts, leading to the production of highly ionizable Na or K gelatinate no longer precipitable with alcohol, and that this ionization should become manifest after the excess of the alkali has been washed away by the repeated perfusion of the powdered gelatin with distilled water.

It would also follow that treatment of the gelatin with $\text{Ca}(\text{OH})_2$ or $\text{Ba}(\text{OH})_2$ should act like the treatment of gelatin with CaCl_2 and BaCl_2 ; *i.e.*, after the base is washed away, the gelatin should remain precipitable no matter what the concentration of the $\text{Ca}(\text{OH})_2$ or $\text{Ba}(\text{OH})_2$.⁷ These demands of the theory are fulfilled (Table III). 1 gm. of the finely powdered gelatin was put for half an hour at about 22°C . into 100 cc. of various concentrations of NaOH , KOH , $\text{Ca}(\text{OH})_2$, and $\text{Ba}(\text{OH})_2$. The gelatin was then put into a funnel,

⁷ The concentrations in which bases can be used are limited by the fact that higher concentrations liquefy the gelatin.

the alkali allowed to drain off, and the gelatin was then perfused four times with 25 cc. of distilled water. After this the gelatin was liquefied and the precipitability of 5 cc. of 1 per cent solution by 95 per cent alcohol was determined at about 20°C. Gelatin which had been previously treated with M/256 or still more dilute KOH or NaOH was precipitable with alcohol, while gelatin treated with M/128 or higher concentrations of these two bases was no longer precipitable

TABLE III.

Nature of base.	Cc. 95 per cent alcohol required for precipitation of 5 cc. 1 per cent solution of gelatin, at 20°C., previously treated for half an hour with one of the following solutions and then perfused four times with 25 cc. H ₂ O.								
	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	H ₂ O
KOH.....	∞	∞	∞	∞	15.9	7.55	6.4	5.25	5.35
NaOH.....	∞	∞	∞	∞	8.6	6.8	6.2	5.5	5.7
Ca(OH) ₂			4.4	5.5	5.3	5.3	5.3	5.9	5.8
Ba(OH) ₂		4.3	4.0	4.0	4.3	4.3	5.0	5.4	5.3

with alcohol, after the excess of base was washed away. The critical concentration is therefore under the conditions of our experiments the same for NaOH and KOH as for NaCl and KCl.

On the other hand, gelatin treated with weak or strong concentrations of Ca(OH)₂ or Ba(OH)₂ did not lose its alcohol precipitability but has the same low alcohol number as gelatin treated with salts of Ca or Ba. No more striking proof of our theory could be expected.

The fact that the alkalies give the same results as the neutral salts confirms our view that the salts combine with the gelatin, forming metal gelatinates, since the combination of alkalies with gelatin can be proved by titration experiments.

III.

Experiments with acids do not contradict our theory but the agreement is not so perfect as in the case of alkalies. According to our theory all strong monobasic acids should render the gelatin non-precipitable at the same concentration (after the acid is washed

away). This seems to be the case, the critical concentration being in the neighborhood of $M/256$ for HCl , HNO_3 , and trichloroacetic acid (Table IV).

The dibasic acids, like H_2SO_4 , should, however, act like diacidic bases, *e.g.* $Ca(OH)_2$, and should not annihilate the alcohol precipitability of gelatin or at least not at low concentrations of the acid. This is true only for H_2SO_4 , and here only partly, but not for the organic dibasic acids, like tartaric, oxalic, and malic. These organic dibasic acids behaved like the monobasic acids (Table IV). The reason for this deviation is not clear. Should it be that the

TABLE IV.

Nature of acid.	Cc. 95 per cent alcohol required for precipitation of 5 cc. 1 per cent solution of gelatin at 20°C., previously treated for half an hour with one of the following solutions and then perfused four times with 25 cc. H ₂ O.										
	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	H ₂ O
HNO ₃		8	8	8	8	8	8	0.7	3.7	4.7	5.1
HCl.....			8	8	8	8	8	1.1	4.5		5.3
Trichloroacetic acid.....	8	8	8	8	8	8	8	0.95	3.7	4.95	5.4
H ₂ SO ₄				8	17.0	12.2	10.2	7.1	1.1	4.0	5.5
Tartaric acid.....	8	8	8	8	8	8	8	6.15	0.9	4.15	5.65
Oxalic acid.....	8	8	8	8	8	8	8	8.75	1.0	3.4	5.05
Malic acid.....	8	8	8	8	8	8	6.8	1.4	1.4	4.3	5.3

organic acids, like oxalic or tartaric acid, and to a lesser extent also H_2SO_4 , form acid salts with gelatin?

The experiments on acids give us an opportunity to compare our results with those of colloid chemists, especially Pauli. Pauli investigated the effects of acid on proteins but in the presence of the acid. As long as low concentrations of acids are used the error is not so great, but with higher concentrations erroneous results are unavoidable. Thus Pauli⁸ states that if acids are arranged according to their power of influencing in moderate concentrations viscosity and precipitability with alcohol they must be grouped in the following order: trichloroacetic, dichloroacetic, sulfuric, nitric, hy-

⁸ Pauli, *Arch. ges. Physiol.*, 1910, cxxxvi, 483.

drochloric, monochloroacetic, and acetic acid. A comparison of this statement with the results on gelatin after the excess of acid is washed away (Table IV) shows that Pauli's statement is no longer tenable.

It might be well to point out that our experiments with acids support in another respect the ionization hypothesis very nicely. In these experiments the gelatin was first treated for half an hour at about 20° with 100 cc. of acid and the latter was then washed away by four perfusions with 25 cc. of H₂O each, as in the experiments with alkalies. The reader will notice that before the gelatin becomes non-precipitable by alcohol it goes through a stage where it can be precipitated with less alcohol than is required for the gelatin not treated with acid. This is due to the fact that gelatin is a stronger acid than base and hence dissociates slightly into H⁺ and gelatin. Since gelatin is only a weak acid it requires the addition of only a trace of a second acid to repress this ionization, and for gelatin treated with HCl and then washed four times with 25 cc. of H₂O this point—the isoelectric point—is reached when the acid used was *m*/512 (Table IV). It was the same for nitric and trichloroacetic acid. For dibasic acids the isoelectric point was at twice the dilution of that in the case of monobasic acids; namely, *m*/1024 (Table IV). If more acid is added, a salt formation between acid and gelatin is produced, the acid combining with a NH₂ group of the gelatin, the gelatin salt undergoing stronger ionization. This action of the acid had been noticed and correctly interpreted by Pauli.⁵ This observation supports the assumption that the non-precipitability of gelatin treated with electrolytes, acids, alkalies, and neutral salts, is due to ionization of the gelatin.

The fact that the isoelectric point is reached at the same hydrogen ion concentration regardless of the nature of the anion of the acid shows that only the former ion influences the result. Exactly the same is true as far as the alcohol precipitability of gelatin by different monobasic acids is concerned, as our Table IV shows.

The writer wishes to call attention to the fact that a 1 per cent gelatin solution near the isoelectric point becomes on standing at not too high a temperature turbid or completely opaque even if no alcohol is added. This only happens when the gelatin has been

treated with acid but not with base or with a neutral salt.⁹ Such naturally turbid isoelectric solutions of gelatin are also characterized by a minimum in viscosity, and a minimum in swelling, all of which tends to support the conclusion that these physical qualities are a function of the degree of ionization of the gelatin.

IV.

Weak acids and bases, *e.g.* acetic acid and NH_4OH , will not cause the gelatin to become non-precipitable. 1 gm. of gelatin was put for 1 hour into beakers containing 100 cc. of different concentrations of these two substances and was afterwards freed from the excess of the solution by four perfusions with 25 cc. of distilled water, as described. It was found that the gelatin so treated remained precipitable by alcohol in all the concentrations used (Table V).

TABLE V.

	Cc. 95 per cent alcohol required for precipitation of 5 cc. 1 per cent solution of gelatin at 20°C., previously treated for 1 hour with one of the following solutions and then perfused four times with 25 cc. H_2O .										
	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	H_2O
Acetic acid.....	6.1	5.5	4.9	4.3	1.8	1.0	0	1.4	3.8	4.7	5.1
NH_4OH	13.5	8.1	6.3	5.9	5.7	5.8	5.5	5.6	5.5		5.4

It is noticeable that the values for acetic acid go through a minimum at M/256 while those for NH_4OH do not show such a minimum, as was to be expected.⁹

The slight effect of acetic acid is not a specific effect of the acetate anion but merely due to the fact that acetic acid is a weak acid, since the weak base NH_4OH shares this peculiarity of the acetic acid.

⁹ It is impossible to bring gelatin nearer the isoelectric point with the aid of salts or bases. For amphoteric electrolytes which are stronger acids than bases the isoelectric point is reached when the dissociation of the H ions is repressed to the level of that of the OH ions, and such a result can only be brought about by the addition of acid to the gelatin solution, but not by the addition of a neutral salt. It is, therefore, not correct to state that a salt like CaCl_2 or NaCl can render a protein like gelatin "isoelectric."

This effect of weak acid and base is a confirmation of our theory, since we assume that electrolytes react in a purely chemical way with gelatin (and proteins in general). Gelatin being a weak acid and a still weaker base, only little gelatin acetate and ammonium gelatin can be formed with acetic acid and NH_4OH respectively. The mass of the new gelatin salt being small at the best, not as many gelatin ions can be formed as are required to render the gelatin non-precipitable by alcohol.

These negative effects of the treatment of gelatin with weak acid and weak base (after the excess of acid or base is washed away with distilled water) are paralleled by equally negative effects on additional swelling and on viscosity.

SUMMARY.

1. The experiments reported in this paper show that if finely powdered gelatin has been treated for 1 hour with the solution of a neutral salt of the type NaCl (univalent cation, univalent anion), and if the excess of salt solution is afterwards washed away with H_2O , a 1 per cent solution of such gelatin in distilled water becomes non-precipitable by alcohol when the concentration of the salt solution used exceeds a certain limit, which is about $\text{M}/128$.

2. When the neutral salt used is of the type Na_2SO_4 (univalent cation, bivalent anion) the critical molecular concentration which renders the 1 per cent gelatin solution under the conditions of our experiments non-precipitable by alcohol is exactly one-half of that for salts of type NaCl ; namely, about $\text{M}/256$.

3. When the powdered gelatin is treated with solutions of neutral salts of the type CaCl_2 or MgSO_4 (*i.e.* bivalent cation, univalent or bivalent anion) and the excess of salt is washed away the 1 per cent gelatin solution in distilled water remains precipitable with alcohol for all concentrations of the salt used.

4. These experiments support the conclusion reached in the former papers of the writer that only the cation of the salt influences the gelatin.

5. The experiments also support the suggestion made in the writer's former papers that the neutral salts act upon proteins, which, like gelatin, are stronger acids than bases, by forming metal

proteinates which dissociate electrolytically into a positive metal and a negative gelatin ion. The metal gelatinates with univalent cation (Li, Na, K, NH_4) are highly ionizable, while those with bivalent cation (Mg, Ca, Sr, Ba) are less or not at all ionizable. The anion of the salt used behaves as if it did not combine with the gelatin at all or as if it entered into a non- or less dissociable bondage.

6. These conclusions are supported by experiments with alkalies showing that if we treat powdered gelatin for half an hour with different concentrations of NaOH or KOH, and then wash the excess of alkali away, a 1 per cent gelatin solution in distilled water made from powdered gelatin thus treated is no longer precipitable with 95 per cent alcohol when the concentration of the alkali used was $\leq \text{M}/128$. When, however, $\text{Ba}(\text{OH})_2$ or $\text{Ca}(\text{OH})_2$ is used the gelatin solution in distilled water remains precipitable by alcohol no matter what concentration of the alkali was used. In this latter case, Ca and Ba gelatinate are formed which according to our assumption are not or little dissociable electrolytically; in the former case, Na and K gelatinate are formed which are highly ionizable. The fact that the alkalies give the same results as the neutral salts confirms our view that the salts combine with the gelatin, forming metal gelatinates, since the combination of alkalies with gelatin can be proved by titration experiments.

7. If we treat powdered gelatin for half an hour with different concentrations of strong monobasic acids, HNO_3 , HCl, trichloroacetic acid, and then wash the excess of acid away, a 1 per cent solution of such gelatin in distilled water becomes (under the conditions of our experiment) non-precipitable with alcohol when the concentration of the acid used was $\leq \text{M}/256$. When we use dibasic acids, the gelatin should remain precipitable by alcohol if the excess of acid is washed away. This is approximately true for H_2SO_4 but is not true for the organic dibasic acids, such as tartaric, malic, and oxalic. A possible explanation of this discrepancy between theory and fact is offered.

8. Weak acid (acetic) and weak base (NH_4OH) act alike inasmuch as neither renders the gelatin non-precipitable (after the acid or base is washed away) which was to be expected if the effect of electrolytes on gelatin is due to a chemical reaction between the two bodies.

THE ORIGIN OF THE CONCEPTION OF PHYSIOLOGICALLY BALANCED SALT SOLUTIONS.

By JACQUES LOEB.

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(Received for publication, April 24, 1918.)

An article published recently in this *Journal* contains the following sentence:

"The recent conceptions of 'physiologically balanced' solutions in their relation to biological phenomena, conceptions enlarged by Loeb and others, complicate the problem considerably by forcing a consideration of the possible antagonistic action of different ions....."¹

This statement is not entirely correct. Herbst had shown that if any of the constituents of the sea water was omitted the larvæ of the sea urchin could no longer develop. From this fact he drew the conclusion that each constituent of the sea water was needed for the development of the larvæ. The writer showed by his experiments on the eggs of *Fundulus*, which are laid and which develop in sea water, that this conclusion was not correct, since: (1) The eggs died very rapidly in a pure NaCl solution of the concentration in which this salt occurs in the sea water, while they could live indefinitely if a small amount of Ca was added to the NaCl; and (2) The eggs developed normally in distilled water, proving that neither Na nor Ca was needed for the development of the eggs.

These experiments, as well as others on the eggs of sea urchins and on jellyfish, led the writer in a paper published 18 years ago to the following conclusion:

"It seems to me that my experiments necessitate the introduction of a new conception, namely, that of *physiologically balanced salt solutions*. By this I mean salt solutions which contain such ions and in such proportions as to completely annihilate the poisonous effects which each constituent would have if it

¹ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 133.

were alone in solution. Sea-water and blood (and approximately a mixture of 96 cc. $5/8$ N NaCl + 2 cc. $10/8$ N CaCl₂ + 2 cc. $5/8$ N KCl) are physiologically balanced salt solutions.

"It will be necessary to investigate how far the conclusions of pharmacologists botanists, and bacteriologists concerning the effects of various salts require a correction on the basis of these new facts and conceptions. Their consideration might even prove of use in problems of immunity and adaptation."²

As far as the writer is aware, the term as well as the conception of "physiologically balanced salt solutions" originated with this statement. In fact there was no reason to suspect the necessity of such a conception until it was shown that marine organisms, of the type of *Fundulus*, whose eggs can live and develop normally in distilled water, are nevertheless sensitive to changes in the relative proportion of the constituents of the sea water.

² Loeb, J., *Am. J. Physiol.*, 1899-1900, iii, 445.

IS THE THEORY OF AXIAL GRADIENT IN THE REGENERATION OF TUBULARIA SUPPORTED BY FACTS?

By MARIO GARCIA-BANUS.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

In his book "Individuality in Organisms," C. M. Child assumes the existence of 'metabolic gradients' in a great number of species of animals and plants, and on this assumption he builds a theory of individuality.

In the case of the hydroid Tubularia, which he uses extensively to prove his theory, the metabolic gradient would lie in the axis of the animal.

The apical end of the metabolic gradient of the major axis is the apical end of the hydranth and from there the rate (metabolic rate) decreases basally through the hydranth. In the stem the metabolic rate is lower than in the hydranth and there is a slight decrease in the basal direction, but at the growing tip of the stolon there is a short gradient in the opposite direction.¹

Child has made no measurements of the rate of metabolism of different regions of the stem of Tubularia. What he really means is that an excised piece of the stem of Tubularia regenerates a new hydranth at the oral end the more rapidly, the nearer this end lies to the original apex of the stem. Such differences he assumes to be due to alleged differences in what he calls 'metabolic rates.' We are therefore only concerned with the question whether the regional differences in the rate of regeneration which Child assumes in Tubularia really exist.

If a piece is cut from a stem of Tubularia a new hydranth will regenerate at each end, and, according to the old experiments of Loeb, the oral end of the piece will, as a rule, regenerate the hydranth

¹ Child, C. M., Individuality in organisms. Chicago, 1915.

sooner than the aboral end. Child in his experiments² cut the stems of *Tubularia* in two or three pieces of equal length, and states that in such cases the oral end of the most apical piece will regenerate a hydranth sooner than the oral end of the most basal piece. This is the actual basis for his theory of 'axial gradient.'

The differences found by Child between the times of emergence of the oral hydranths of the two pieces are, however, so slight that the suspicion arises that they may be merely within the limits of error and individual variation.

As a matter of fact, only in one single case of those that he presents, is there a marked difference between the time of the regeneration of the oral hydranths of the apical and basal pieces. And in this case the average is based on the observation of only eight individual stems. The apical pieces give an average time of emergence of the oral hydranths equal to 27 hours, while for the basal pieces the average is 36.5 hours. In all the other cases which he gives, the differences are far from being so marked. Thus in one case the averages for the hydranth formation of eight stems were 38 hours for the apical pieces and 39 hours for the basal pieces, while in a third case the averages were 42 for the apical pieces and the same number 42 for the basal ones! In the case of the stem cut into three pieces of equal length the averages for the oral hydranth formation are for one of the series of twenty stems, 42.5 for the most apical piece and 44 for the most basal one, and in another series of ten stems the averages are 117 for the apical piece, 118 for the middle piece, and 120 for the most basal one.

The differences in all these experiments, except the first, are so small that they may well lie within the limits of variation and of error of such experiments. In order to be sure we repeated Child's experiments to try to ascertain the foundation for his conclusions.

The experiments were carried out at New York (with material from Long Island Sound and from the Lower Bay) and at Woods Hole, and the results were similar in both places. The material and conditions, especially at Woods Hole, were so excellent that we were

² Child, C. M., An analysis of form-regulation in *Tubularia*. IV. Regional and polar differences in the time of the hydranth formation as a special case of regulation in a complex system. *Arch. f. Entwicklungsmech.*, 1907, 24, 1.

able to keep the regenerating stems alive in finger bowls for more than fifteen days.

In each case we recorded the time of emergence of the hydranth outside the perisarc, and observations were made at least every two hours.

TABLE 1.

Time in Hours Required for Emergence of the Oral Hydranths of Two Pieces of Equal Length.

NUMBER OF INDIVIDUAL	APICAL PIECE	BASAL PIECE
1	75	71
2	81	73
3	71	77
4	71	71
5	77	77
6	71	71
7	95	71
8	95	79
9	81	71
10	71	71
11	71	73
12	71	—
13	79	73
14	83	95
15	71	73
16	95	71
17	77	95
18	75	79
19	71	83
20	71	73
Averages.....	77.6 hours	76.1 hours

In the first group of experiments (table 1), the stems were cut into two pieces of equal length; several series of this type, with twenty stems each were made with similar results, namely, that *there is no marked difference between the times of appearance of the oral hydranths of the two pieces*. Very often the oral hydranth of the basal piece is the first to appear. As an example of this may serve the experiment recorded in table 1. The size of both pieces was 20 mm., so that they were long enough to show a marked difference according

to Child's opinion. Of the twenty stems included in this experiment, in eight of them the oral hydranth of the apical piece appeared first, but there were seven cases in which the oral hydranth of the basal piece was the first to appear and four cases in which they both appeared at the same time. The averages of the time of appearance are 77.6 hours for the oral hydranths of the apical pieces and 76.1 hours for the oral hydranths of the basal pieces. The difference is even slightly in favor of the oral hydranth of the basal piece, but it is so small that it is within the limits of error or of individual variation.

In a second group of experiments the stems were cut into three pieces of equal length. The pieces in this case were each 10 mm. long and the results are similar to those of the other series.

The experiment in table 2 may serve as an example. There are in this case ten stems in which the oral hydranth of the third and most basal pieces emerges before the corresponding hydranths of the most apical pieces, while there are only five stems in which the oral hydranth of the most apical piece is the first to appear and there are two cases in which both appear at the same time. If we compare the apical piece with the middle piece, the results are the same; in seven cases out of the twenty, the oral hydranth of the middle piece is the first to appear, while there are only two cases in which the contrary happens, and six cases in which both appear at the same time.

The averages in this experiment are: 78.6 hours for the oral hydranths of the apical pieces, 77.8 for those of the middle pieces, and 74.2 for those of the basal pieces. This shows again that the slight difference in the time of appearance turns out to be in the opposite sense of what we ought to expect from Child's theories. We have made several series of this type with similar results, and in some cases the differences in favor of the basal pieces were even greater than in the case just shown. For instance, in one of these experiments in which twenty stems were cut into three pieces of 10 mm. each, the averages for the time of emergence of the oral hydranths were in hours: for the apical piece 92, for the middle piece 84, and for the basal piece 76. We have chosen the experiment, the results of which are given in table 2, as an example, because in it the individual differences are not very great, so that the data are more con-

stant and the averages more reliable. It is therefore a mere matter of chance whether the oral hydranths of the apical or basal pieces emerge first, as long as both pieces have equal length.

Comparing pieces of different size from the same stem, Child found that the shorter pieces will form hydranths a little later than

TABLE 2.

Time in Hours Required for the Emergence of the Oral Hydranths of Three Pieces of Equal Length, from the Same Stem of Tubularia.
Length of the Pieces = 10 Mm.

NUMBER OF INDIVIDUAL	APICAL PIECE	MIDDLE PIECE	BASAL PIECE
1	91	69	67
2	77	71	69
3	71	77	73
4	69	91	71
5	67	69	67
6	69	66	67
7	71	71	73
8	73	73	71
9	—	—	79
10	79	69	73
11	75	75	77
12	79	73	73
13	73	73	75
14	—	91	91
15	91	91	79
16	—	95	71
17	—	91	69
18	91	73	73
19	91	91	75
20	91	69	91
Averages.....	78.6 hours	77.8 hours	74.2 hours

the longer ones, but he states that since the more basal pieces will form the oral hydranths later than the more apical, the pieces must be cut in such a way that the shorter piece is always the more apical one. Thus he says the two factors of size and level are opposed to each other instead of acting in the same sense and adding their effects, as would be the case if the shorter piece were basal to the larger one.

We have found the existence of this factor of the size of the pieces to act constantly but irregularly, and we have tested the other factor of level comparing a series of stems cut in such a way that the shorter piece is the more apical with another series of stems, under the same

TABLE 3.

Time in Hours Required for Emergence of the Oral Hydranths in Pieces of Different Length.

Ratio of the length of the pieces = 1:2.

Series A Apical piece = 10 mm. Basal piece = 20 mm.

Series B Apical piece = 20 mm. Basal piece = 10 mm.

SERIES A			SERIES B		
Number of individual	Apical piece 10 mm.	Basal piece 20 mm.	Number of individual	Apical piece 20 mm.	Basal piece 10 mm.
1	67	67	1	67	67
2	53	53	2	67	67
3	55	55	3	53	69
4	55	67	4	55	69
5	55	55	5	67	97
6	53	55	6	—	67
7	67	67	7	79	97
8	—	67	8	91	67
9	67	67	9	91	71
10	73	67	10	67	67
11	75	67	11	59	71
12	67	55	12	91	91
13	55	67	13	59	67
14	71	67	14	67	67
15	69	67	15	67	67
16	91	67	16	67	67
17	95	73	17	67	67
18	53	66	18	77	67
19	67	67	19	67	67
20	55	67	20	53	67
Averages.....	65.4	64.2		69.0	70.3
Differences.....	1.2 hours			1.3 hours	

conditions and cut into the same proportions, but in which the shorter piece is the more basal one.

The results are given in tables 3 to 5. In the experiment recorded in table 3 the ratio of the lengths of the pieces was 1:2. We took

two series of twenty stems each; in series A the shorter piece of 10 mm. was the apical one and the longer of 20 mm. was the basal one; while in series B, on the contrary, the shorter piece was the basal

TABLE 4.

Time in Hours Required for Emergence of the Oral Hydranths in Pieces of Different Length.

Ratio of the length of the pieces = 1:3.

Series A Apical piece = 10 mm. Basal piece = 30 mm.

Series B Apical piece = 30 mm. Basal piece = 10 mm.

SERIES A			SERIES B		
Number of individual	Apical piece 10 mm.	Basal piece 30 mm.	Number of individual	Apical piece 30 mm.	Basal piece 10 mm.
1	99	73	1	79	79
2	69	69	2	67	71
3	69	67	3	71	73
4	67	67	4	77	77
5	69	69	5	71	69
6	67	67	6	71	69
7	67	67	7	79	91
8	67	67	8	91	91
9	69	69	9	91	91
10	73	71	10	67	77
11	69	69	11	69	73
12	67	67	12	77	77
13	91	71	13	67	71
14	71	71	14	67	71
15	77	71	15	77	77
16	69	69	16	69	71
17	77	67	17	73	73
18	67	67	18	71	91
19	67	67	19	69	71
20	69	71	20	77	79
Averages.....	72.0	68.8		73.9	77.1
Differences.....	3.2 hours			3.2 hours	

one and the longer the apical one. The averages are in series A: for the apical piece (10 mm.) 65.4 hours, and for the basal piece (20 mm.) 64.2 hours; in series B: for the apical piece (20 mm.) 69.0, and for the basal piece (10 mm.) 70.3 hours. The oral hydranth of the larger

piece emerges about an hour earlier than that of the smaller pieces a difference that is very small indeed but the same in both series. There was no influence of the level factor.

TABLE 5.

Time in Hours Required for the Emergence of the Oral Hydranths in Pieces of Different Length.

Ratio of the length of the pieces = 1:4.

Series A Apical piece = 10 mm. Basal piece = 40 mm.

Series B Apical piece = 40 mm. Basal piece = 10 mm.

SERIES A			SERIES B		
Number of individual	Apical piece 10 mm.	Basal piece 40 mm.	Number of individual	Apical piece 40 mm.	Basal piece 10 mm.
1	72	72	1	70	94
2	70	70	2	76	82
3	94	74	3	74	82
4	72	72	4	74	70
5	74	70	5	82	78
6	94	94	6	94	74
7	74	78	7	74	82
8	98	74	8	94	74
9	118	70	9	72	74
10	70	70	10	—	—
11	82	96	11	72	82
12	64	54	12	—	—
13	98	94	13	70	70
14	82	106	14	70	74
15	70	82	15	72	82
16	94	96	16	70	94
17	—	74	17	70	78
18	102	72	18	76	—
19	94	94	19	72	82
20	70	70	20	70	94
Averages.....	83.8	79.1		75.1	80.0
Differences.....	4.7 hours			4.9 hours	

In the experiment recorded in table 4, the ratio of the lengths of the pieces was 1:3. In series A the shorter piece of 10 mm. is the apical one and the longer piece of 30 mm. is the basal one, while in series B the reverse occurs. The averages are in series A: for the

short apical piece 72.0 hours, and for the longer basal piece 68.8 hours; in series B: for the longer apical piece 73.9, and for the shorter basal one 77.1. The differences between the time of regeneration of the shorter and the longer pieces are 3.2 hours in favor of the larger piece in both series.

In table 5 the ratio of the sizes of the pieces was 1:4 and the averages are in series A: for the shorter apical piece 83.8 hours, and for the longer basal one 79.1 hours, with a difference of 4.7, while in series B the averages are: for the longer apical piece 75.1 hours, and for the shorter basal piece 80.0, with a difference of 4.9.

In any of these cases there was no evidence of the existence of level or regional differences on the stems of *Tubularia*.

CONCLUSIONS.

Child has based his theory of the 'metabolic gradients' on the assertion that if a stem of *Tubularia* is cut into two pieces the oral end of the apical piece will regenerate a hydranth more rapidly than the oral end of the basal piece. The differences in the time of regeneration observed by him were so small that they seemed to lie within the limits of error of such experiments.

The writer repeated Child's experiments and found this suspicion justified. The rate of regeneration of the oral hydranth of an apical piece is on the average identical with the rate of regeneration of the oral hydranth of the basal piece. If in one series the average is in favor of the apical piece, in another the reverse may be found.

There is no evidence of the existence of level or regional differences of the rate of regeneration in the stem of *Tubularia*, and as a consequence there is no basis for the theory of 'axial gradient' in this species.

I have to acknowledge my thanks to Dr. Jacques Loeb, who has suggested and directed the present work.

PNEUMONIA AT A BASE HOSPITAL.

BY RUFUS COLE, M.D., AND W. G. MACCALLUM, M.D.

Contract Surgeons, U. S. Army.

Early in February, a commission was sent by Surgeon-General Gorgas to Texas to study the pneumonia existing there. This commission was composed of Rufus Cole, W. G. MacCallum and Oswald T. Avery, Contract Surgeons, U. S. Army, Captains A. R. Dochez and R. A. Kinsella, and Lieutenants F. G. Blake, T. M. Rivers, H. John, F. A. Stevens and Wm. C. Von Glahn, Medical Reserve Corps. This special study was undertaken at the base hospital, Fort Sam Houston, San Antonio, Texas. This hospital was chosen for this work merely because pneumonia was prevailing there to a large extent at the time, and also because of its proximity to the laboratory of the Southern Department, where facilities were available for carrying out the bacteriologic and pathologic examinations. It was thought that any facts disclosed by the study there might be applicable to other camps and base hospitals. Any general application of our observations, however, must be made with reservations, for it is possible that the conditions in other hospitals may be different from those found here, and therefore that the pneumonia existing in other hospitals may differ in essential details from that present in this one.

Our preliminary survey of the cases in the hospital made it evident that not all the cases were of the same variety and that our present knowledge did not permit a ready differentiation of the cases of the different kinds. An extensive statistical study was therefore out of the question, and we decided to make a very careful clinical, bacteriologic and pathologic study of a limited number of cases.

The study was commenced, Feb. 1, 1918, and continued about six weeks. This paper is composed of extracts from the preliminary reports made by us to the Surgeon-General.

PART I. ETIOLOGY AND CLINICAL FEATURES OF THE PNEUMONIA OCCURRING IN THE HOSPITAL.

TOGETHER WITH NOTES CONCERNING THE PLACE AND MODE OF INFECTION IN THE CASE OF POSTMEASLES BRONCHOPNEUMONIA.¹

BY RUFUS COLE, M.D.

Varieties of Pneumonia.

The account of the results of our observations will be rendered more intelligible if we state briefly that we have found two distinct varieties of pneumonia in the wards of this hospital. First, there are present cases of acute lobar pneumonia, which are essentially identical with cases of this disease occurring in civil life. With all these cases pneumococci have been associated; second, there are present a large number of cases of bronchopneumonia, which differ essentially from the cases of the former disease in clinical features, pathology and etiology. The majority of these cases occur as complications or sequelae of measles, though they may undoubtedly follow other infectious diseases. The pathology of these cases is carefully described by Dr. MacCallum in his report. The etiologic agent in all the cases studied here has appeared to be a streptococcus producing lysis of red blood corpuscles when grown on a medium containing blood. Similar observations have also been made in other Army hospitals, and it seems probable that these streptococci are responsible for most of the bronchopneumonia occurring in the present epidemic of measles. Previous observations, however, suggest that other bacteria may also be responsible for cases with similar clinical and pathologic features. In the further discussion, we shall refer to these two varieties of pneumonia as "acute lobar pneumonia" and "bronchopneumonia." Finally, there occurs, in no inconsiderable number of patients, a combination of these two types of disease, or infection with both pneumococci and streptococci. In these cases, one or the other of the two varieties of disease is probably primary, secondary

¹ This part of the work was carried out with the assistance of Oswald T. Avery, A. R. Dochez, R. A. Kinsella, F. G. Blake, T. M. Rivers, H. John, and F. A. Stevens.

infection later occurring with the subsequent development of the other variety.

Since the regular hospital data were not considered sufficient for our purposes, complete histories were obtained from a series of cases of both varieties, and our own physical examinations were made and the results carefully recorded. Thirty cases of bronchopneumonia, thirty-two cases of acute lobar pneumonia, and nine cases in which the two varieties of infection were present have been studied.

It was impossible to study all of the cases of pneumonia in the hospital in this way. Consequently, in a considerable number of cases coming to necropsy, the clinical data are slight. On the other hand, many of the cases studied by us ended in recovery, and consequently the pathologic lesions present can only be inferred from the knowledge obtained in the other cases in which necropsy was performed. In twelve cases of bronchopneumonia, however, the clinical observations could be correlated with the pathologic findings.

Bronchopneumonia.

Bacteriology.—The evidence that *Streptococcus haemolyticus* is the etiologic agent in these cases is given by the study of cultures made from the blood during life and from the organs and tissues of the body after death, and by the study of the sputum coughed directly from the lung.

Cultures from the blood during life were made in fifteen cases. In only two cases was a growth obtained. In these cases a pure growth of *S. haemolyticus* occurred. The cultures were made only within twenty-four hours of death, however. Studies of the bacteria present in specimens of sputum expectorated from the lung were made in all the cases. The method employed was practically that described previously for the study of the sputum in lobar pneumonia.

Specimens of sputum directly coughed from the lung were obtained in sterile containers. After the washing of portions of this sputum, cultures were made on the surface of blood agar plates, and a small portion of sputum was injected into a mouse. After the death of the mouse, the peritoneal exudate was examined microscopi-

cally, and cultures were made from this exudate and from the heart's blood.

In all the cases that we have classified as bronchopneumonia, the cultures from the sputum on blood agar plates showed the presence of *S. haemolyticus* in large numbers. In most of the cases in which the mouse test was carried out, examination of the heart's blood showed the presence of this organism (in sixteen out of seventeen cases). In thirteen of the specimens of sputum *Bacillus influenzae* as well as *S. haemolyticus* was found on the plates. In five instances, the patients died and came to necropsy, and in these instances *B. influenzae* was also present in the affected lung. In eight instances, *B. influenzae* was also present in the heart's blood of the mouse. Whether this organism plays any part in this disease is not known. Its very frequent presence is of much interest.

Cultures were made from the infected areas in the lungs, the heart's blood, the pleura, the pericardium, and frequently from other organs of the patients coming to necropsy. In making cultures from the lungs, the pleura over the infected area was seared with a hot knife, and a cone-shaped piece of the lung was removed with sterile instruments. Cultures and smears were then made from the tip of the portion removed. In all of the cases diagnosed as bronchopneumonia during life and all of those cases coming to necropsy which showed the presence of the lesions described by Dr. MacCallum as interstitial bronchopneumonia, *S. haemolyticus* was found to be present in the affected portion of the lung, usually in pure culture. In most of the cases in which empyema occurred, these organisms were present in the fluid in pure cultures.

It was also the rule to find these organisms in the heart's blood after death. In a few cases influenza bacilli were also present in the blood and tissues, along with *S. haemolyticus*. These cases, however, did not differ from those in which the streptococcus was present alone.

Clinical Course.—The picture of the clinical course of the disease which can be constructed from our data is necessarily incomplete and possibly incorrect in certain details. It is hoped, however, that it may be amplified and corrected by more complete studies in other camps. The study of these cases has not made it possible to differ-

entiate, clinically, between the cases showing the lesions described by Dr. MacCallum as interstitial bronchopneumonia, and those showing the lesions described by him as lobular pneumonia. Consequently, in our discussion all these cases are termed "bronchopneumonia."

Onset.—All the cases of this group have followed measles. In seven of our thirty cases, a history was obtained of a chronic cough, coryza or sore throat previous to the appearance of the symptoms of measles. Whether pneumonia is more likely to occur in those men who have had previous infections of the respiratory tract is not certain, but it is possible that this factor is of some, but not paramount, importance.

A considerable number of cases of measles show laryngeal infection as shown by the occurrence of hoarseness and huskiness of the voice. That this is a true inflammatory lesion is shown by the fact that minute ulcerations may be present on the vocal cords.

The first definite symptoms of the disease are usually cough, fever, slight respiratory distress, and the expectoration of a mucopurulent sputum. All of these symptoms, as is known, usually occur in some degree during the febrile stage of measles. In certain cases of measles, the temperature does not fall with, or shortly after, the appearance of the rash, as is usually the case, but the fever continues and becomes higher and the symptoms mentioned increase in severity. In other cases the temperature falls to normal, and then after a period of from a few days to three weeks the temperature again becomes elevated and the symptoms mentioned become severe and characteristic of the disease. In none of the cases we have seen, however, nor in any of those of which we have histories, has there been a complete disappearance of all the symptoms referable to the respiratory tract during the interval between recovery from measles and onset of bronchopneumonia. Even in the cases in which the onset has occurred following the discharge of the patient from the hospital, it is quite certain that cough or respiratory difficulty was present at the time of discharge and persisted up to the time of onset of the pneumonia.

In all the cases that we have observed, the onset has been gradual. In no case was there a chill with sudden elevation of temperature. Vomiting during the early period has been rare.

These observations as well as the pathologic studies, indicate that the infection, as well as the lesion, is probably a descending one, and that no sharp line can be drawn either in time or symptoms, between the occurrence of the pharyngitis, laryngitis, bronchitis, and finally the bronchiolitis and bronchopneumonia.

Symptoms.—When the disease is well developed, the following are the common symptoms:

Fever: Usually the fever is not high, rarely going higher than 104°F., and even in the uncomplicated cases the temperature is frequently irregular. Wide diurnal sweeps of the temperature curve, however, have usually been associated with the presence of empyema.

Pulse: The pulse has not been extremely rapid, even in the cases near death. Special attention has not been given to the occurrence of irregularities.

Respiratory Distress: This is the most marked and characteristic symptom of the disease, and is usually present even in the early stages. This is quite distinct from the respiratory difficulty seen in acute lobar pneumonia, with the expiratory grunt. In bronchopneumonia the great difficulty is with inspiration, the accessory muscle then being strongly brought into action. Frequently the expansion of the chest wall is slight, but the diaphragmatic pull is powerful, the costal margin being drawn on, and the intracostal spaces being markedly retracted with each inspiration. The patients seem consciously to have difficulty in getting sufficient air into the chest, but the inspiratory phase is not prolonged. The respiratory rate is usually not extremely rapid; frequently, even in very ill patients, the rate is not over thirty per minute.

Cyanosis: This is practically always present, even in the early stages, and in the more severe cases becomes extreme.

Cough: The cough is troublesome; frequently it is markedly increased by change in posture.

Sputum: Usually the expectoration is fairly free and moderate in amount. Its character varies markedly in the different cases and in the different stages of the disease. Early in our studies it was thought that the sputum had a specific appearance, being light-greenish, mucopurulent, sometimes somewhat blood streaked, and of a homogeneous character, spreading like molasses over the bottom of a cup. Later, however, typical cases have been observed in which this kind of sputum was never seen. In some cases the sputum has been nummular, quite mucoid, the masses being tenacious and somewhat sticky. In other cases the nummular masses have been greenish and very purulent. In none of the uncomplicated cases has the sputum been sticky and of a rusty color like that seen in acute lobar pneumonia, nor has it contained large amounts of bright-red blood, as is sometimes seen in the early stages of lobar pneumonia. Whatever its character, it practically always contains large numbers of pus cells.

Restlessness: This is a striking feature of the condition. The patients are fairly alert, rarely delirious, but always anxious and frequently frightened. Sleeplessness, probably due largely to the cough and respiratory difficulty, is very frequently present.

Pain: This is frequently one of the symptoms, and is due to the pleurisy which is almost invariably present. Abdominal pain, in the uncomplicated cases, has not been frequent in this series.

In the typical cases, the symptoms we have mentioned are quite striking and more or less characteristic. It must be remembered, however, that similar symptoms, though of milder grade, are also present in cases of bronchitis without involvement of the smaller bronchioles. The symptoms are characteristic enough in the severe cases, however, to differentiate them from cases of acute lobar pneumonia.

Signs.—We have already spoken of the obvious signs of respiratory distress and the cyanosis which is so striking. Physical examination of the chest may or may not reveal signs indicating consolidation. This, of course, depends on the intensity and focal distribution of the lesions. Where the process is very diffuse, the chest may be resonant throughout, on percussion, though in certain regions, especially at the bases, the percussion note is frequently impaired. On auscultation, râles are usually heard throughout the chest. There are frequently musical and squeaking, in addition to moist, crackling, râles. In typical cases, the râles are more numerous during inspiration, and the inspiratory murmur is harsh. In such cases, the expiratory murmur may be scarcely audible; even though the expiratory murmur is inaudible, however, a succession of medium, moist râles may be heard during the expiratory phase. Over the areas of impaired resonance the breath sounds may be very faint or absent until after the patient coughs, when harsh, mucous râles are heard, and the breath sounds become audible.

In some cases characteristic signs of consolidation, impaired resonance, tubular breathing and intensified spoken and whispered voice sounds are present over small areas. Frequently these signs persist for a few days, and then entirely disappear. In our early cases, these signs were most confusing and difficult to interpret. As we became better acquainted with the pathologic lesions, however, the

meaning became obvious. I shall not attempt to bring the signs into relation with the lesions, since this relation becomes quite clear on the reading of Dr. MacCallum's report.

In no uncomplicated cases have we seen wide areas of dulness with characteristic tubular breathing and other signs of consolidation. When these signs have been present, there was always a complicating lobar pneumonia. In a few cases, however, later in the disease, there has been impairment of the percussion note over a wide area, with quite distant tubular breathing over this area, but with voice sounds fairly well transmitted. The repeated insertion of a needle in these cases has failed to reveal fluid, and the needle has felt as though it were in a more or less solid lung. We have interpreted these signs as due to a quite widespread involvement of the lung, with a marked degree of proliferation and plugging of the bronchi, as described by Dr. MacCallum. These are probably the cases which go on to necrosis and abscess formation, such as was seen in three of the necropsy cases. It must be remembered, however, that in the only cases of this type we have seen at necropsy there was a marked accumulation of fluid in the pleura.

Complications.—Focal infections, such as tonsillitis and otitis media, may be present. They have not occurred frequently in this series, however. The most important complication is empyema.

Empyema.—This condition occurs with great frequency, being present in sixteen of the thirty cases, and in all but one of the necropsy cases. In ten of the cases, the condition was diagnosed by aspiration of fluid from the chest during life. In four additional cases, fluid was obtained from the chest on aspiration, but in these cases, the fluid was only slightly turbid, and contained no bacteria demonstrable by culture.

The diagnosis of the presence of fluid or pus in the pleural cavity in these cases is frequently difficult. In the cases in which large accumulations of fluid have occurred, no great skill is required. It is probably of great importance, however, to detect the presence of small amounts of fluid, and to do this as early after the fluid appears as possible. Small amounts of fluid can be detected only by employing the methods of physical examination of the chest. Roentgen examination is of assistance in certain cases. We have already

stated that occasionally, over the more intensely affected areas, there is dulness on percussion, and the breath sounds may be distant. To determine whether these signs are due to an intrapulmonary lesion or to the accumulation of fluid in the pleural cavity is frequently very difficult. The greater intensity of the dulness on percussion, when fluid is present, and the distant faint tubular breathing heard at the margin of the area, are the signs of greater importance. Fortunately, the decision can be obtained by insertion of a needle, and in doubtful cases, this may be done not only once, but many times, if necessary.

Character of the fluid: In most of our cases, the fluid obtained has been thin, but turbid, owing to the presence of bacteria and numerous fibrinous, purulent floccules. In only three or four cases has a thick, greenish-yellow pus been encountered. The infrequency of an exudate of this character should lead one to suspect, when such fluid is found, that the condition is a complication of lobar pneumonia and not of bronchopneumonia.

Mortality.—Of the thirty patients studied by us, fourteen died and sixteen recovered, a mortality of 47 per cent. Several of the patients are still very ill, however, and will probably die. Since the cases were not taken in succession, it is impossible to say whether or not this is a fair estimate of the mortality rate. Probably the percentage of cases ending fatally in this hospital has been considerably higher than this. A most striking fact is that all the necropsy cases in our series were complicated by empyema. In many cases it has seemed that death was related directly to this complication.

Treatment.—No observations were made on the effect of any special form of treatment. Since the occurrence of empyema is an important factor in the outcome of the treatment of this complication, it deserves a few words of discussion.

Seven of the empyema patients were operated on, a rib resection with drainage being performed in each case. Of these patients, four have died. All of the others are very sick, and a fatal outcome is probable in one or more. On the other hand, of four patients in whom fluid containing streptococci was aspirated from the chest and operation was not performed, all have died. There were three cases in which fluid containing a moderate number of pus cells but no bac-

teria was aspirated from the chest. One of these patients was operated on; the other two were not. All three of these patients have lived, and from present indications will probable recover, though the patient operated on is still very ill. Our observations are not sufficient to enable us to draw any conclusions as to operation in this condition. They suggest, however, that in the cases in which fluid containing streptococci is aspirated from the lung, the chest should be opened and drained. But the question of operation in these cases is a difficult one, and we cannot be guided entirely by our experience with empyema complicating lobar pneumonia. The problem deserves special study.

Lobar Pneumonia.

During the course of our study of cases of bronchopneumonia, a considerable number of cases of typical lobar pneumonia were encountered. Some of these were chosen for study deliberately in order to have a control for the other work; in some the differential diagnosis was made only after careful investigation. One purpose of this study was to demonstrate to the hospital and laboratory personnel the fact that, under the conditions existing here, rapid and accurate diagnosis of the type of pneumococcus causing the infection in lobar pneumonia could be made. A small number of the patients were treated with serum by us or under our direction. This was also done for the purpose of demonstration.

Thirty-two cases of lobar pneumonia were studied. Only two of these patients had recently had measles: one one month, and the other six weeks previous to onset. There seemed no definite relation between the measles and pneumonia in these cases. In one other case, not included in this series, an apparent pulmonary infection with pneumococcus Type IV occurred during convalescence from measles. There was a pleural exudation of clear, sterile fluid; but the signs of lobar pneumonia were never definite.

Of these cases, seventeen were associated with Type I pneumococcus in the sputum; three with Type II; four with atypical Type II; one with Type III; six with Type IV; and one with *Streptococcus mucosus*.

The etiologic diagnosis was made within twenty-four hours in all these cases except two. In these cases, the delay was due to the fact that the sputum was very scanty and unsatisfactory, and the tests had to be repeated. The examination in both of these cases was made late in the disease. (Some of the patients were studied on admission, others only after they had been in the wards for some days.) In seventeen cases, the diagnosis was made within eight hours, the Avery medium being employed. Considering the conditions under which we worked, this result seems quite satisfactory and demonstrates the possibility of type determinations in a military hospital.

In eleven cases, the presence of fluid in the chest was determined by aspiration. In five cases the fluid was clear, or very slightly turbid. In four of these cases the fluid was sterile; in the remaining case the fluid was contaminated after removal from the chest and cultures could not be made. In these five cases, the fluid was removed by aspiration, and all the patients recovered without operation. In five other cases, the fluid was very turbid, and cultures revealed the presence of pneumococcus Type I. All of these patients were drained. Three of them are now well or are satisfactorily convalescing. Two of them are still very ill and have irregular fever. In the remaining case the fluid was thick and green, and cultures showed the presence of pneumococcus Type II. This patient was operated on and is also convalescing.

In eight of the Type I cases, the patients were treated with serum. Two of the treated patients were found to be sensitive to horse serum, and it was necessary to desensitize them before the administration of large amounts of serum. All but one recovered promptly; this one developed empyema. This patient has done well since operation and is now convalescent. The results of this small series of cases, therefore, were satisfactory.

Of the thirty-two patients, two died; two of the empyema patients are still ill, and the result is in doubt. The others have recovered.

This experience with lobar pneumonia in this hospital leads us to believe that it does not differ essentially from that seen in civil hospitals, except, perhaps, in its relative mildness. This, however, is to be expected in a population composed of healthy young adults.

Cases of Mixed Infection or Doubtful Cases.

In addition to the cases which were clinically identified as bronchopneumonia and which at necropsy were found to show the lesions described as interstitial bronchopneumonia or lobular pneumonia, and the cases which were typically lobar pneumonia, both clinically and at necropsy, there occurred a series of cases which were more or less complex in their clinical, etiologic and pathologic features.

There were seven cases in which evidence was obtained of a mixed infection with pneumococcus and *S. haemolyticus*, the infections being present simultaneously or in succession. Four of these cases were in patients who had recently had measles:

The first patient had signs of bronchopneumonia beginning a few days after measles. Four days later there was an acute exacerbation of symptoms with signs resembling pneumonia. This patient developed empyema on the side of the lobar involvement; and from this fluid during life pneumococcus Type IV was obtained. Death came six days after the onset of the severe symptom. The necropsy showed the presence of lobar pneumonia of the left lower lobe and an area of typical bronchopneumonia in other portions of the lung. From the blood and the right lung, *S. haemolyticus* was isolated. From the pleural exudate both *S. haemolyticus* and pneumococcus Type IV were cultivated. That a combined infection in this case was present is certain. The chronologic course of events is more difficult to decide. It is impossible to say definitely whether the patient had a bronchopneumonia, with secondary pneumococcus infection, which led to septicemia and death, or whether the streptococcus infection occurred later, or finally whether the two infections occurred simultaneously.

The second patient had otitis media and also pulmonary symptoms lasting almost a month following measles. There then occurred an exacerbation of signs and symptoms, and the ward surgeon made a diagnosis of pneumonia. We saw him two days later and found an empyema on the left side, in addition to some signs of consolidation of the left lower lobe. The fluid from the chest contained *S. haemolyticus*, but the blood culture showed the presence of pneumococcus Type IV. The patient died two days later, and at necropsy a true lobar pneumonia was present, but no evidences of bronchopneumonia. In the cultures at necropsy, only *S. haemolyticus* was obtained from both lungs, and *S. haemolyticus* and *B. influenzae* from the blood. This case is difficult to interpret. The patient had a lobar pneumonia, and this was probably due to pneumococcus Type IV, although for some unexplained reason these organisms were not found at necropsy. Whether the streptococcus infection occurred early in the illness or only shortly before death cannot be determined. Unfortunately, the early clinical notes are incomplete, and we have no knowledge of the severity of the early lung involvement.

The third patient developed fever and respiratory symptoms ten days after an attack of measles. In the interval, however, he had had slight, irregular fever. We saw him four days later. He had then only signs of diffuse lung involvement, with indications of empyema on the left side. The sputum contained *S. haemolyticus*, as did also the purulent fluid aspirated from the chest. No pneumococci were isolated. Four days later he was operated on, and the pleural cavity was drained. He lived almost two weeks, and then died. At necropsy an undrained pocket of pus was found between the left lung and the pericardium, and in addition there was almost complete uniform consolidation of the entire left lung. The other lung showed no evidences of bronchopneumonia. At necropsy, *S. haemolyticus* was found in all the cultures, as well as a gram-negative bacillus producing a putrefactive odor. In this case lobar pneumonia existed without the presence of pneumococci being demonstrated at any time. This may have been due, however, to the overgrowth of the culture with a putrefactive organism, which was undoubtedly a terminal invader. When the lobar pneumonia began, or whether bronchopneumonia was ever present, cannot, of course, be decided.

The fourth patient developed typical lobar pneumonia during convalescence from measles. The blood culture showed the presence of pneumococcus Type I. He developed fluid in the left chest which was purulent, and contained pneumococcus Type I and *S. haemolyticus*. He died three days after the tapping, and at necropsy no pneumonia was found, but several small abscesses in the left lung and a very large amount of purulent exudate in the left pleura. The necropsy culture showed only the presence of streptococci. No pneumococci grew.

There were three patients suffering from acute lobar pneumonia in the pneumonia wards who gave no history of measles but in whom evidence of additional infection with *S. haemolyticus* was obtained. In two of these cases the pneumococcus causing the lobar pneumonia was Type II; in one, Type I.

One of these patients, during convalescence from pneumonia due to pneumococcus Type II, developed empyema, and the pleural exudate contained *S. haemolyticus* and *B. influenzae*. He had had some cough and sore throat for three weeks before the onset of pneumonia, and it is possible that the streptococcus infection antedated the pneumonia. There is no definite evidence for this, however.

The second patient had quite a typical attack of acute lobar pneumonia, also due to pneumococcus Type II. He gave no history of any pulmonary symptoms previous to the onset of pneumonia. During convalescence, he developed an irregular fever and signs of scattered lesions in the chest. There occurred an effusion of fluid in the pleura, cultures from which were sterile. The examination of the sputum at this time, however, revealed the presence of *S. haemolyticus*, which had previously been absent. In spite of the fact that the chest

fluid was sterile, the chest was opened and drained. The signs of diffuse involvement of the lungs, however, continued, and the patient, one week after operation, is still seriously ill. The study of this case is incomplete, and the evidence of secondary streptococcus infection is not satisfactory, but is suggestive.

The third patient suffered from acute lobar pneumonia, due to pneumococcus Type I, involving the right lower lobe. Convalescence was normal for a week. Signs of pulmonary involvement persisted, however. At the end of the week, the temperature again became elevated, and the presence of fluid in the chest was suspected. Repeated insertion of a needle, however, failed to reveal fluid. The sputum, however, changed in character, and examination now showed the presence of *S. haemolyticus* and *B. influenzae*. Finally, about two weeks after the second onset of fever, a small amount of dark, foul-smelling fluid was obtained by puncture, apparently from the lung. This showed the presence of *S. haemolyticus* and the staphylococcus. About a week later the chest was opened and a lung abscess was drained. Whether the occurrence of abscess was due to secondary infection with streptococci or whether this infection was secondary to the abscess formation is, of course, not certain.

There were two additional patients seen by us in the pneumonia ward who developed empyema, with *S. haemolyticus* in the fluid. One patient had never had measles and the other had had measles two weeks previously. In the case without measles, the occurrence of pneumonia was doubtful.

This patient had been slightly ill for two weeks previous to admission, but had been performing his duties. The pleural effusion was present on admission, so infection occurred outside the hospital. The sputum contained *S. haemolyticus* and *B. influenzae*, and the pleural fluid contained *S. haemolyticus*. The patient was operated on and recovered.

In the second case, which followed measles, there were signs of lobar pneumonia with empyema. The sputum contained only *S. haemolyticus* and *B. influenzae*. The pleural exudate contained *S. haemolyticus*. The patient was operated on, and his present condition is fairly favorable. It is possible that this patient had bronchopneumonia, and not lobar pneumonia.

These cases indicate the frequency of complicated pulmonary infections in the present epidemic, and they also show the difficulty of interpreting the course of events in certain cases. It is true that in most of these cases no careful observations were made during the early stages of the illness. It seems quite certain that lobar pneumonia may sometimes occur in patients already infected with streptococci and with bronchopneumonia already present in the lungs.

That bronchopneumonia occurs secondarily to lobar pneumonia is not so well shown by our cases; but they do show that in cases of lobar pneumonia, secondary infection with streptococci not infrequently occurs. This is obviously of much practical importance, suggesting that, so far as possible, patients with lobar pneumonia should be protected from infection with this streptococcus.

Place and Mode of Infection in Postmeasles Bronchopneumonia.

From the data previously presented, there appears little doubt that a hemolytic streptococcus is the chief, if not the only, cause of the bronchopneumonia following measles which occurs among the soldiers at this post.

We have previously indicated that the infection in these cases is probably descending, occurring first in the throat, and successively involving the lower respiratory passages, and finally the lung. It therefore became of much interest and practical importance to learn whether hemolytic streptococci are present in the upper respiratory tract of all measles patients, and, if so, at what stage of the disease they are first present, or, on the other hand, whether these bacteria are present only on the throats of these patients who later develop pulmonary lesions.

We therefore first attempted to determine the prevalence of hemolytic streptococci in the throats of all the patients in the measles wards of this hospital. To do this, cultures were made from the pharynx and tonsils of these patients on blood-agar plates. The plates were incubated twenty-four hours and then studied for the presence of hemolyzing streptococci. These organisms were identified by isolation in pure culture from a single colony and by testing staining reaction, morphology, cultural characteristics, bile solubility, hemolytic activity and fermentation reactions. Hemolysis was determined by testing the power of a twenty-four hour broth culture to hemolyze an equal amount of a 5 per cent. suspension of the red blood cells of rabbits. The degree of hemolysis was recorded at the end of two hours at 37°C. Table 1 gives the results of the study.

TABLE 1.

Incidence of Streptococcus Haemolyticus in Throats of Patients in Measles Wards.

Total Number of Cases Examined	Number of Positive Cases	Number of Negative Cases	Per Cent. of Positive Cases
69	39	30	56.5

At the time of the examination, these patients had been in the measles wards for periods of time varying from one to fifty-five days. Of the sixty-nine patients examined, thirty-nine, or 56.5 per cent., showed the presence of *S. haemolyticus* in the throat.

These observations indicate that a large proportion of the patients confined to the measles wards harbor these organisms in the throat.

It next appeared of importance to learn whether or not this high incidence of carriers of hemolytic streptococci is peculiar to the measles wards or whether a similar state of affairs exists among the patients in other wards of the hospital. The incidence of streptococci of this variety in the throats of normal individuals under ordinary circumstances has not been determined accurately. In the presence of an epidemic of streptococcus infection, such as streptococcic sore throat, it has been found that a considerable number of healthy persons may carry this organism in the throat. On the other hand, the observers who have made fairly extensive studies on the flora of the normal throat, in the absence of epidemics of this kind, report that the occurrence of actively hemolytic streptococci in the throats is very rare. At the time of our arrival at San Antonio, it was evident that an epidemic of coryza, laryngitis and mild bronchitis existed among both the civil and military population. In some limited observations which we made, it was found that in the throats of persons suffering from this affection, hemolytic streptococci, and also influenza bacilli were occasionally present, singly or combined. It was possible, therefore, that the study of persons other than those suffering from measles might show a high incidence of hemolytic streptococci in the throats. Consequently cultures were made from the throats of patients in a ward in which the patients were suspected of having tuberculosis but who had no other disease. Table 2 presents the results of this study.

TABLE 2.

Occurrence of Streptococcus Haemolyticus in the Throats of Tuberculosis Suspects (Ward 27).

Total Number of Cases Examined	Number of Positive Cases	Number of Negative Cases	Per Cent. of Positive Cases
28	6	22	21.4

The occurrence of *S. haemolyticus* in the throats of these patients is considerably less than the incidence among the measles patients. It should be mentioned that the patients in the tuberculosis ward are not rigidly isolated from patients in other parts of the hospital, transfers from this ward to another, and vice versa, not infrequently being made. Several cases of tonsillitis and pharyngitis were discovered during the process of making cultures. Moreover, these patients live in very close association, being confined in the ward and only a few of them being confined to bed.

There were two methods available to discover whether the measles patients acquired the hemolytic streptococci before admission, or whether transfer from one patient to another probably occurred in the ward. One method was to make cultures on a large number of men in the barracks from which the patients with measles came, with the object of learning whether the incidence of streptococci in the throats of these healthy men was less than that among the men in the wards; the other was to make cultures from the patients with measles on admission to the hospital and then to repeat the examinations from time to time, in order to learn whether or not any of the patients with negative cultures later acquired the organisms. The latter method was chosen as consuming less time and more likely to give definite information. The results of the study are presented in Table 3.

TABLE 3.

Occurrence of Streptococcus Haemolyticus in the Throats of Measles Patients on Admission, and the Subsequent Acquisition of the Organism in the Cases Previously Negative.

		Per Cent.
Total number of cases examined.....	44	
Number of cases positive on admission.....	5	11.4
Number of cases positive 3 to 5 days after admission.....	17	38.6
Number of cases on final examination—8 to 16 days after admission.....	25	56.8

An analysis of this table indicates that a relatively small percentage of patients on admission to the measles wards harbor *S. haemolyticus* in their throats. However, as the time of residence in the ward increases, the number of carriers of *S. haemolyticus* increases, until finally the percentage of incidence becomes as high as that found in the measles wards in general. Because of these observations, there seems to be very little reason to doubt that a large percentage of measles patients acquire *S. haemolyticus* in their throats for the first time during their stay in the hospital wards.

While this study is not extensive enough to be absolutely conclusive, it indicates strongly that the high incidence of bronchopneumonia during convalescence from measles is directly related to the transfer of the infectious agent from one patient to another in the wards of the hospitals.

These observations led us to suspect that the frequent occurrence of streptococcus complications, especially empyema, might also be related to the possible wide distribution of hemolytic streptococci among the patients in the pneumonia wards. Although an effort has been made to admit to the wards assigned to the care of patients suffering from acute lobar pneumonia only patients suffering from this disease, it is quite certain that owing to the difficulties of diagnosis, a considerable number of patients with bronchopneumonia have been treated in these wards. We have already referred to certain of these cases.

To investigate this question, cultures were made from the throats of all the patients in two wards which contained only patients thought to have acute lobar pneumonia. Cultures were made from forty-five patients. The technic employed was exactly that used in the tests previously mentioned. It was found that twenty-six, or 57.7 per cent., of all these patients harbored hemolytic streptococci. In view of the high incidence of streptococcus infections among the lobar pneumonia patients, these results are most interesting and suggestive. There is no reason to believe that the percentage of patients carrying hemolytic streptococci on admission is larger than the percentage of measles patients who do so when admitted. This question, however, has not been investigated.

COMMENT AND CONCLUSIONS.

The studies indicate that the cases of pneumonia at the base hospital, Fort Sam Houston, are chiefly of two varieties: first, acute lobar pneumonia, which does not differ essentially from that which occurs elsewhere; and second, bronchopneumonia, which in most cases, at present at least, follows measles.

The pulmonary lesions in most cases of this type of bronchopneumonia are characteristic and specific and have been studied and described by Dr. MacCallum. The etiologic agent in all the cases studied by us has been *S. haemolyticus*. There is no evidence presented by this work that indicates that pneumococcus causes the lesions and symptoms of this condition. Pneumonia following measles may be due to pneumococci, but the pulmonary lesion is then of the lobar variety. This complication of measles, however, is comparatively rare. Cases may occur in which both types of infection and both types of lesions are present. The sequence of events in such cases is difficult to determine and is probably not always the same.

Streptococcus infections following lobar pneumonia occur with considerable frequency in this hospital. Bronchopneumonia similar to that following measles may also probably occur as a sequel of acute lobar pneumonia, though the evidence for this is not conclusive. Whether in the cases of streptococcus empyema, complicating lobar pneumonia, pulmonary lesions due to the streptococci are always present or not, has not been determined.

The mortality in the cases of bronchopneumonia is very high; that of uncomplicated lobar pneumonia is low. Practically all the fatal cases of bronchopneumonia are complicated by empyema. The incidence of empyema among the uncomplicated cases of lobar pneumonia does not seem to be extremely high.

Our observations indicate that the number of measles patients harboring hemolytic streptococci on admission to the hospital is not large. The majority of the patients with measles acquire this organism during their stay in the hospital. The chance of developing postmeasles streptococcus infections is therefore increased by residence in this hospital.

A very large number of the patients suffering from acute lobar pneumonia have hemolytic streptococci in their throats. We have no direct evidence that they acquire these bacteria in the hospital, but the presumptive evidence indicates that many of them do so.

The work indicates that the high incidence of pneumonia in this hospital, and the resulting high mortality, has been due, to some extent at least, to infection occurring within the hospital itself. The conditions are not unlike those surrounding puerperal fever and surgical wound infections. While in measles, raw surfaces do not exist on which infection can occur, this disease renders the respiratory mucous membrane especially vulnerable to infection with streptococci. Possibly in other diseases, as scarlet fever and even lobar pneumonia, similar conditions exist. When infection is once started in a ward in which the patients are closely associated, the streptococci become widely distributed; they probably gain in virulence with repeated transfer through the human subject, and serious and widespread infection results.

Probably the conditions in this hospital are not unique. It is possible that the widespread incidence of fatal pneumonia in the other army hospitals may have a similar explanation.

PART II. PATHOLOGY.²

By W. G. MacCALLUM, M.D.

The material for the following study was derived from thirty-seven necropsies performed on the bodies of patients who died in the base hospital of Fort Sam Houston, Texas. They were selected as cases of pneumonia, but in three of them it proved that death had resulted from other causes. Nevertheless, the lungs in these patients, as well as those from several other miscellaneous cases in which the necropsies were performed by the resident staff, were studied.

The results are by no means easy to analyze, since there are many complications, many combinations of different infections, and many variations arising from differences in the stages of the disease at which the patient died. Briefly stated, however, it appears that two main

²This part of the work was carried out with the assistance of Lieut. W. C. Von Glahn, M. R. C., U. S. Army.

types of bacterial infection are concerned. On the one hand, there are cases due to infection with one or another form of the pneumococcus; on the other hand, many cases have occurred in which a hemolytic streptococcus is the etiologic agent. Other bacteria have been found, notably the influenza bacillus, a gram-negative bacillus of undetermined nature, and occasionally one or another of the staphylococci; but all of these appear to be rather accidental invaders, and it seems scarcely probable that they are in any way responsible for the main anatomic changes.

Analysis of the cases appear to show fairly conclusively that the pneumococcus is responsible for those in which lobar pneumonia was found. The *Streptococcus haemolyticus*, in most instances, seems to cause a peculiar form of bronchopneumonia, which on account of its anatomic characters I have designated "interstitial bronchopneumonia." There are, however, some cases in which this organism, growing in overwhelming numbers or with especial virulence, produces a patchy pneumonia of a type more closely resembling the familiar lobular of bronchopneumonia found so often as a terminal event in persons dying of some chronic disease, or in those in whom aspiration of infected material has occurred. This may be referred to as "lobular pneumonia."

One of these infections may be superimposed on the other, and there may even be found lesions corresponding to each in the same lung.

Fibrinopurulent pleurisy with abundant exudate has occurred with extreme frequency in these cases.

Since it is recognized that pneumonia frequently follows measles, this relation was especially studied. There were fifteen definite cases of measles in the series, and in eleven of these the interstitial bronchopneumonia was found. Two showed, at necropsy, lobar pneumonia alone, two lobular pneumonia alone, while two presented a combination of lobar and interstitial bronchopneumonia. In all there were seventeen cases of interstitial bronchopneumonia, and in the six cases of this condition not definitely following measles, no history of measles was obtained in five, although it was conceded that in the course of the great local epidemic these men might have passed through mild attacks of measles which were not men-

tioned when the clinical history was taken. The sixth case was at first regarded as measles, but later as scarlet fever. From all the cases of interstitial bronchopneumonia the hemolytic streptococcus was isolated, and it seems clear that this is the true causative factor, whether it gains entrance on the basis of a predisposing measles or otherwise. Details concerning all these relations will be given in full in a later report, but in this preliminary report it is necessary to describe the following conditions as they occurred in this series: (1) interstitial bronchopneumonia; (2) lobular pneumonia; (3) lobar pneumonia; (4) empyema; (5) combined infections; (6) other complications.

1. Interstitial Bronchopneumonia.

This is the condition already fairly well known through the work of Bartels, Delafield, Steinhaus, Hecht and others as occurring in children as a sequel of measles, whooping cough, etc. Their descriptions correspond closely with what we have found in adults, except in the general lack of complicating empyema in children, and in certain minor histologic details, notably the presence in the lungs of children of extraordinary giant cells derived from the epithelium. None of these writers determined the nature of the bacteria concerned. On the other hand, although it has been recognized by various workers in this country that the hemolytic streptococcus is to be found in the bronchopneumonia following measles, none of them seems to have determined the exact nature of this peculiar bronchopneumonia. The association of the hemolytic streptococcus with the interstitial bronchopneumonia must, therefore, be especially emphasized.

The term "interstitial bronchopneumonia" was chosen as expressing briefly the salient features of the lesion. It may not be the most satisfactory term possible, because it fails to describe accurately the earliest stages, and leaves out of account the process of organization of the exudate which is very common; but any term that could describe the whole course of a progressive process would be unwieldy.

The interstitial bronchopneumonia has been studied in various stages in different cases and found to produce extraordinarily different appearances as it progresses.

In the earliest stage, the pleural surface of the lung is smooth and glistening. The lung is, in general, air containing, although atelectatic patches may be making their appearance. On section, small hemorrhagic foci are found scattered through the lung, each showing, as a rule, a gray rather opaque center. These foci measure from 2 to 3 mm. in diameter, sometimes more, and are so small that several may occur in one of the secondary lobules of the lung, that is, in one of the lobules marked off by the interlobular septa (W. S. Miller). Microscopically, it is found that these foci represent the ends of the bronchioles together with the adjacent alveoli. The bronchiole and the ductulus alveolaris are filled with leukocytes, among which streptococci are found in pairs or in short chains. There is some infiltration of the bronchial wall with leukocytes, and the adjacent alveoli contain a few leukocytes, occasional streptococci, coagulable fluid, and great numbers of red blood corpuscles. Not only the alveoli which form a continuation of the bronchiole, but also those which lie near its wall, seem to be affected.

In a somewhat later stage the lung can still be distended with air, although the patches of collapse are more extensive. On section it is found to be studded throughout large areas with small, gray nodules which project above the cut surface like miliary tubercles, and are often surrounded by a red or grayish halo. At this time there may be visible a minute cavity or depression in the center of each which marks the lumen of the bronchiole. This may be represented, however, by the opaque contents of the bronchiole. These nodules have been mistaken by more than one for miliary tubercles, and it seems conceivable that the peculiar appearance of this and later stages may be in part, at least, responsible for the almost universal statement that measles is commonly followed by tuberculosis. Microscopically, such nodules are found to consist of a bronchiole filled with exudate of leukocytes, sometimes, but not often, associated with fibrin. The epithelial cell layer lining the bronchus is partly disintegrated or detached. The bronchial wall is hyperemic and thickened largely by the infiltration into its crevices of numbers of mononuclear wandering cells which have replaced the leukocytes. The alveoli about the bronchiole appear to contain less blood at this stage; only those immediately continuous with the ductulus alveolaris contain poly-

morphonuclear leukocytes; the others about the bronchiole usually contain a network of fibrin with mononuclear cells. The alveolar walls in the immediate neighborhood of the bronchiole are thickened by an infiltration of mononuclear cells (lymphocytes, plasma cells and larger wandering cells). Surprisingly few streptococci are found, and those chiefly in the bronchial exudate. Fibrinopurulent pleurisy, often with excessive effusion of greenish, turbid fluid, accompanies the process from this stage on.

In a still later stage the lung is usually much collapsed, dark blue, flabby, and airless except in the anterior portions. This is produced chiefly by the pressure of the pleural exudate, but partly by the occlusion of the bronchioles. At this stage, shot-like nodules 3 or 4 mm. in diameter may be felt all through the lung. On section, the pasty, airless lung sinks into a concave surface, leaving the gray peribronchial nodules projecting conspicuously. The interlobular septa have by this time become greatly thickened and infiltrated with cells and fibrin so that they stand out most conspicuously as whitish-yellow lines, marking out the whole lobulation of the lung in polygonal fields. In each of these fields there may be three or four projecting nodules which now usually show distinctly a central bronchial lumen. The surrounding tissue may be fairly dense, so that the peribronchial thickening is marked out chiefly by its opaque whiteness. Hemorrhage may in some cases stain the outlying regions about the nodules. If the bronchi be opened with the scissors, they are found to be slightly dilated toward the periphery of the lung, where they become thick walled as they run into the terminal portion which forms the center of the nodule. The contents are thick and glutinous. Microscopically, such a lung shows a very great infiltration of the bronchial wall with masses of mononuclear cells. The epithelium usually still persists in places, although much of it is desquamated. The lumen is filled with exudate of leukocytes with rather few chains of streptococci. Sometimes all the lining of the bronchus has disappeared so that in cross section it appears like an abscess. The walls of the adjacent alveoli are greatly widened and stuffed with mononuclear cells, desquamated epithelium, fluid and rather dense plugs of fibrin. Further out the alveolar walls are still thickened and infiltrated, and the alveoli contain chiefly fluid and

desquamated epithelium. At this stage, organization of the exudate in the bronchi and the alveoli is usual. Indeed, this organization occurs with surprising rapidity, so that it may be quite advanced in patients who have apparently been ill only about ten days or two weeks. In the bronchi the new connective tissue and blood vessels arising from several points in the wall pervade the exudate and form richly vascular columns of fibrous tissue which extend into the alveoli and branch into each one. The lymphatics in the walls of the bronchi and blood vessels and in the interlobular septa are distended with mononuclear cells and fibrin, and contain numerous bacteria. The bacteria apparently reach the pleural network in this way, and this seems to be the most plausible explanation of the infection of the pleura. The interlobular septa and perivascular tissues are densely infiltrated with wandering cells and become very conspicuous. The pleural surface is covered with a thick, shaggy layer of fibrin, and the pleura itself is greatly thickened by being converted into a vascular granulation tissue which is gradually replacing the fibrin.

Streptococci are present in great numbers on the surface of the fibrinous exudate, as well as in the purulent fluid in the cavity of the pleura. It is to be noted that they, unlike the pneumococcus, are not to be found scattered everywhere in the meshes of the fibrin. It seems possible that they may digest and destroy the fibrin, but at any rate they are found only on its free surface. They are not engulfed by phagocytes as freely as are the pneumococci, and they are found less frequently in the tissues themselves. Even when in an inflamed tissue, such as the lobulated, fat masses which project into the pleura from the outside of the pericardial sac, streptococci are found in a matted layer over the free surface, they can be traced down into the crevices between the lobes of fat only so far as these are freely open. If the surfaces adhere no bacteria are found in the obliterated depth of the crevice.

In still later stages, more extensive infiltration of the peribronchial tissue occurs, and solid yellowish patches from 1 to 2 cm. in diameter appear. The induration about these, with edema and hemorrhage, becomes confluent, so that quite large areas may appear consolidated. As to the nature of the healing process and recovery from this change, we have had no opportunity to learn anything.

In one case in which in some parts of the lung the lesions just described occurred in a moderate stage of advancement, other areas showed a great increase in the number of streptococci, with a wide dissemination into the tissue and a correspondingly intense inflammatory reaction. In other cases, much later, after a long course in which empyema of long standing has been drained by operation, the lung has been found to contain extensive abscesses, sometimes confluent into large, purulent areas. In such cases, and in these alone, one may expect to find secondary lesions in distant organs. It is rather remarkable that this particular hemolytic streptococcus seems to be an organism of rather slight virulence, with no tendency to spread through the body. Septicemia occurs only in the hours just before death, if at all, and in only one protracted case was there found an infarct-like lesion in the spleen. In all the others, the abdominal organs were normal.

2. *Lobular Pneumonia.*

In these cases there was evidently an overwhelming infection with a virulent organism, or what amounts to the same thing; the patient offered no resistance to the invasion. The lungs present irregular, patchy, hemorrhagic areas of consolidation which are not especially peribronchial or limited in size. In them the streptococci are found in amazing numbers, often in long, tangled chains, scattered through the alveolar contents as well as in the exudate in the bronchi. In this respect the condition contrasts with that found in the interstitial bronchopneumonia. The exudate itself is chiefly composed of polymorphonuclear leukocytes, with some blood. There is nothing especially characteristic about this process, but it does occur after measles and is sometimes associated with the formation of areas of necrosis of the whole tissue which subsequently assume the appearance of abscesses. These are really not typical abscesses, but rather necrotic areas of consolidated lung tissue loaded with great numbers of bacteria. There were four of these cases.

3. *Lobar Pneumonia.*

Thirteen cases of lobar pneumonia occurred in this series and presented the well known anatomic picture which need not be described. In ten of them the pneumococcus was found, but in the remaining three it was missed, apparently because it was overgrown by the hemolytic streptococcus or by other organisms, since all of these were protracted cases with empyema, in which death occurred some days after operation and drainage of the open pleural cavity.

In four cases, pneumococcus Type IV was isolated. Two showed the presence of Type I and two Type II. In two the type remained undetermined.

It seems very probable that in all the cases the lobar form of pneumonia was caused by the pneumococcus. In those in which the pneumococcus was not obtained in culture, organisms morphologically like them and having the characteristic distribution of the pneumococcus are seen in the sections of the affected lung.

Secondary infection with the hemolytic streptococcus seems to have occurred in some cases, although it remains difficult to tell whether the streptococcus may not have preceded the pneumococcus. In two cases in which the pneumococcus seemed to predominate at necropsy, it was thought probable that the streptococcus infection had been followed by that with the pneumococcus. On the other hand, four or five cases showed the streptococcus in great numbers at necropsy, while the pneumococcus was found only in such lesions as a vegetation on the heart valve or an abscess in the rectus muscle. In one case the pneumococcus was found only in the pleural fluid. In another it had been present in the blood before death. In such cases it seemed probable that the pneumococcus had been outgrown and replaced by the streptococcus.

Of the thirteen cases, five showed the pneumococcus alone. In none of these was there any empyema or excessive outpouring of fluid pleural exudate. But of the remaining eight cases in which there was also a streptococcus infection, there was empyema in all except one, in which both pleural cavities were obliterated by old adhesions.

Certain histologic features with regard to the distribution of pneumococci may be mentioned. While in the case of the interstitial bronchopneumonia the streptococci are in most cases present in relatively small numbers and then confined chiefly to the exudate in the lumen of the bronchus and the immediately adjacent alveoli, to the contents of the lymphatic and the surface of the pleural exudate, the pneumococci in lobar pneumonia are present in great numbers and are scattered diffusely through the whole lung and everywhere through the pleural exudate. It is true that there are some cases of intense infection in which the streptococci grow in immense numbers throughout the affected portions of the lung; but ordinarily they are not to be found, or only rarely seen in the fibrinous exudate of the outlying alveoli. In the lobar pneumonia the pneumococci are rather more abundant in the bronchioles and ductuli alveolares than in the more peripheral alveoli; but many are to be found intimately mingled with the network of fibrin in all the alveoli and in the pleural exudate. The activity of phagocytes appears to be far greater in the case of the pneumococcus, so that great numbers of them are found enclosed in the leukocytes, while most of the streptococci appear to lie free in the exudate.

The transportation of the organisms by way of the lymphatic channels, in the walls of the blood vessels, and interlobular septa and pleura is striking in the case of the pneumococci, as well as of the streptococci. It seems probable, although not proved, that it is in this way that the pleura becomes infected. Injections of the lymphatics from the pleural network gave beautiful preparations showing the connections of this network with the enormous, deep lymphatics which run toward the hilum of the lung, in the walls of the bronchi and blood vessels, and in the septa. These aided in the recognition of the relation of the bacteria to the lymph channels.

4. *Empyema.*

Empyema occurred in twenty-six of the thirty-seven cases of the series. In every case in which there was empyema, *S. haemolyticus* was demonstrated, and although empyema occurs in pure pneumococcus infections, only those cases in this series in which there was

also a streptococcus infection developed actual empyema. The exudate appears quite early in the cases of interstitial bronchopneumonia, and is usually a thin, turbid, greenish fluid with floating shreds of fibrin and a relatively thin, fibrinous covering over the lung. Usually this fibrinous exudate cannot be readily peeled off, but is intimately adherent to the lung and partly organized. Very large amounts of fluid may accumulate—in one instance several liters. The effect is, of course, largely mechanical, causing the collapse of the lung, with corresponding cessation of its function; but it seems that the presence of such a huge culture of streptococci, which appear to grow in enormous numbers in a position in which they are removed from actual contact with the tissues, must be a serious menace to the patient. Further studies should be made with regard to the toxic properties of this fluid and to the presence of bactericidal substances there.

After operation, the pleural cavity tends to become infected with various organisms which confer an extremely foul odor on the exudate. Encapsulated pockets of purulent fluid are often found between the lobes, even though the main cavity may have been fairly well emptied.

5. Combined Infections.

Mention has already been made of the fact that both the pneumococcus and the streptococcus may occur in the same individual, and clinical study may show definitely which of these infections appeared first and which was superimposed. It is difficult to arrive at any conclusion as to this from the anatomic conditions, for the ordinary criteria of the duration of an inflammatory process—organization of exudate, production of mononuclear infiltration and indurative new growth of tissue in the framework of the lung—appear so rapidly in the interstitial bronchopneumonia and are so delayed in lobar pneumonia that they help but little.

In three cases there has been found in the lungs a definite intermixture of the anatomic lesions of lobar pneumonia with those of interstitial bronchopneumonia. These conditions seem to tend to exclude each other to some degree, even when they occur side by side in the same lung; but in some places the characteristic lesions of in-

terstitial bronchopneumonia are found embedded in the uniform consolidation of a lobar pneumonia, emerging clearly into view as one leaves the area of lobar consolidation and passes over into the rest of the lung. In two cases the pneumococcus was recovered from the area of lobar consolidation, the streptococcus from the uncomplicated foci of interstitial bronchopneumonia in the same lung. These organisms are, however, too much alike, morphologically, to allow one to recognize them with certainty, in stained sections, in association with their specific lesions, when they occur together.

6. Other Complications.

Complications with regard to other organs are rare in the cases of infection with this hemolytic streptococcus. Pericarditis occurred a few times, but most of the cases of pericarditis were associated with pneumococcus infection. Otitis media is recorded in several cases. There were two cases of acute nephritis, probably not dependent on this infection, since the kidneys in all other cases were normal. On the whole, there seems to be a very striking absence of involvement of any organs other than the lungs.

The range of complications in the case of pneumococcus infection has been rather greater. There were four cases of pericarditis, one of peritonitis, one of symmetrical bilateral abscess of the rectus abdominis muscles, and one in which death occurred suddenly in convalescence, from embolic occlusion of the pulmonary arteries.

SUMMARY.

In the Army camps represented in this hospital, measles has been prevalent. Pneumonia occurred frequently, but not always as a sequel to measles. The nature of measles is unknown, and we have had no opportunity to learn what anatomic changes measles alone can produce. But it does produce coryza, conjunctivitis and laryngitis, and these conditions appear to predispose to infection of the respiratory tract with bacteria. This predisposition is made evident by the great proportion of the cases of the series in which streptococcus infection followed measles; but it is evident that streptococcus infection may occur in a person who has not had measles, and it is

quite probable that other diseases, such as scarlet fever, predispose to its entrance in the same way as measles. When *S. haemolyticus* gained a foothold, it usually caused in this series of cases the anatomic complex called here "interstitial bronchopneumonia." This is the same whether it is preceded by measles or scarlet fever, or by no other disease, and its characters are due to the specific effects of the streptococcus. When lobar pneumonia followed measles, the pneumococcus was in this series accompanied by the streptococcus, and in some cases the lobar pneumonia was complicated, anatomically, by the corresponding bronchopneumonia.

Infection with the hemolytic streptococcus does not always cause an interstitial bronchopneumonia, but may produce a patchy lobular pneumonia.

One of the most interesting features of our study of the cases in this hospital is the recognition of the invariable connection of *S. haemolyticus* with that characteristic anatomic lesion which was well known, but to which we have for convenience given the name "interstitial bronchopneumonia." This lesion is easily recognized in all its stages by its gross appearance, since the prominent, gray, solid peribronchial nodules with surrounding edema, hemorrhage, organization and induration bear no resemblance to areas of pneumonic consolidation, which are homogeneous, solid patches, on the cut surface of which a plug of exudate projects from each alveolus. The microscopic appearance, as described above, is equally specific and characteristic, and there is no possibility of confusion with lobar or lobular pneumonia.

Empyema is a practically constant accompaniment of this condition, and is of extremely serious import.

The organism does not seem to be very virulent, and there is little tendency for it to enter the blood stream or to produce complicating lesions in distant organs.

Lobar pneumonia due to the pneumococcus has been found in many cases, not especially related to the occurrence of measles, but often complicated by secondary or coincident infection with *S. haemolyticus*. Its characters are exactly as seen elsewhere.

A FURTHER STUDY OF ETHYLHYDROCUPREIN (OPTOCHIN) IN THE TREATMENT OF ACUTE LOBAR PNEUMONIA.*

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In a previous communication¹ we reported on a series of thirty-two cases of lobar pneumonia due to pneumococci and treated with ethylhydrocuprein (optochin) hydrochlorid. By means of bactericidal tests of the patient's serum in vitro the absorption and elimination of the drug in these cases were studied. It was concluded that the hydrochlorid of the drug is rapidly absorbed from the gastro-intestinal tract into the circulating blood; that when an amount of the hydrochlorid represented by 0.024 to 0.026 gm. per kilogram of body weight of the patient is administered by mouth per twenty-four hours, the blood serum of the patient acquires the property of destroying pneumococci in the test tube; that the best way to insure the rapid production and maintenance of this bactericidal action in the blood is to divide the total amount of the drug in such a way that the first dose is relatively large and is followed at intervals of not more than three hours by smaller equal doses. For example, if the patient is of average size and is to receive 1.5 gm. in twenty-four hours, he is given a first dose of 0.45 gm., and this is followed by seven doses of 0.15 gm. each at regular intervals. It was further shown that during administration of the drug the pneumococci in the body may become "fast" or resistant to a considerable concentration of ethylhydrocuprein.

The purpose of the present paper is to present the data accumulated in an extension of the former work and to give the final results of two years' experience in the treatment of lobar pneumonia with ethylhy-

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1. Moore, H. F., and Chesney, A. M.: THE ARCHIVES INT. MED., 1917, 19, 611.

drocuprein. It may be stated that the experimental results obtained during the first year have been for the most part confirmed by the study of cases treated during the second year, and in addition some new facts have been ascertained which it is desirable to record. The experimental observations will be recorded first, and then the entire series of cases will be analyzed from a clinical standpoint.

The method of investigation described in the previous paper has again been used and has been found satisfactory. Briefly, this consisted in repeated determinations of the bactericidal power of the patient's serum at 37.5 C. for young (four to six hours) broth cultures of pneumococci. The strains used were stock strains and usually the type of pneumococcus employed for any case differed from that causing the infection. Almost all the patients admitted to the wards of the Hospital of The Rockefeller Institute from October 1915 to May 1917, suffering from lobar pneumonia due to pneumococci belonging to Groups II, III and IV, were treated with ethylhydrocuprein. Cases due to pneumococci of Group I did not receive ethylhydrocuprein, but were treated with specific immune serum. A limited number of patients infected with pneumococci of Groups II and III were treated with ethylhydrocuprein by mouth and in addition received type homologous immune serum intravenously, or concentrated "extract" of such serum subcutaneously or intravenously.

Of the forty-three patients treated with ethylhydrocuprein during the season 1916-1917, two received the base (optochin base) by mouth; forty received the hydrochlorid by mouth, and one patient received the hydrochlorid intramuscularly at first and later by mouth. In Table 8 are presented the details as to dosage of the drug, clinical features and so forth, in these forty-three cases.

Administration of Ethylhydrocuprein Base by Mouth.

The serum of the patients (Nos. 2786 and 2783) who received the base by mouth failed to show either bactericidal activity or power temporarily to inhibit the growth of pneumococci, although the amount of the drug given should have been sufficient to produce such a result if the base were as readily absorbable as the hydrochlorid. The difficulty of absorption from the gastro-intestinal tract is undoubtedly dependent on the fact that the drug in this form is very slightly soluble.

"Fastness" of Pneumococci to Ethylhydrocuprein.

In our former communication we reported one instance in which the infecting pneumococcus became "fast" to ethylhydrocuprein in the human body. A further example of this phenomenon has been observed in the present study. The details follow:

Hosp. No. 2825.—A street cleaner, aged 50; weight 69 kg.

Past History.—Unimportant.

Present Illness.—Cough for one day; chill and bloody expectoration on day of admission.

Status on Admission.—Patient dyspneic, cyanotic and decidedly ill; temperature 105.2 F.; pulse 120; respirations, 32; dulness with suppression of breath sounds and numerous fine moist râles over left lower lobe. Leukocyte count 15,000. Sputum tenacious, hemorrhagic and containing *Pneumococcus* Type II. Blood culture showed *Pneumococcus* Type II, three colonies per cubic centimeter.

Course and Treatment.—Ethylhydrocuprein hydrochlorid started by mouth on second day of disease; schedule as follows in periods of twenty-four hours: first period, 1×0.6 gm. + 8×0.15 gm. (0.026 gm. per kilo of body weight per twenty-four hours); second period, 12×0.15 gm.; third period, 11×0.15 gm.; last period of eighteen hours, 7×0.15 gm. + 2×0.1 gm. Blood culture taken thirty-six hours after the commencement of the ethylhydrocuprein treatment showed twenty-four colonies of *Pneumococcus* Type II per cubic centimeter of blood and the patient's general condition seemed worse at that time. On the following day the blood culture showed sixty colonies per cubic centimeter and on the last day of life blood culture showed 700 colonies of *Pneumococcus* Type II per cubic centimeter of blood. The patient became progressively worse and died on the fifth day after admission to the hospital. Judging from physical signs, spread of the infected area in the lungs did not take place. The total amount of ethylhydrocuprein given was 6.5 gm. No toxic symptoms referable to the drug were observed.

Samples of blood serum of this case were obtained before beginning treatment and eight times during the course of treatment; these samples were tested for pneumococcal power against a four-hour-old stock culture of *Pneumococcus* Type I. *Pneumococci* grew unhindered in the specimen obtained before beginning treatment, but the tests of the specimens obtained later showed that the ethylhydrocuprein conferred the usual degree of pneumococcal power on the serum as early as 5.5 hours after the first dose was administered and that this pneumococcal power was maintained throughout the treatment.

The specimens of patient's serum showing pneumococcal power were pooled and the effect of the pooled serum on two strains of pneumococci, obtained from the patient's blood, was studied; one of these strains was obtained from the patient's blood on admission to the hospital and before the ethylhydrocuprein

treatment was started and the other strain from his blood two hours before death. The results are given in Table 1. The technic employed was that previously described,¹ using 0.001 c.c. of a four-hour-old broth culture to inoculate 3 c.c. of serum. The serum was previously heated at 56 C. for one-half hour. The bacterial count of the plates is given in approximate figures.

TABLE 1.
Bactericidal Tests of Hospital No. 2825.

Serum	Strains of Pneumococcus Obtained from Blood	Number of Colonies of Pneumococci per 0.5 C.c. of Serum When Plated*		
		Immediately	After 1½ Hours Incubation	After 24 Hours Incubation
Before ethylhydrocuprein was started	On admission (before ethylhydrocuprein)	400	2,000	Confluent
	Two hours before death	400	2,000	Confluent
Pooled specimens obtained during ethylhydrocu- prein treatment	On admission (before ethylhydrocuprein)	400	300	0
	Two hours before death	400	2,000	Confluent

* Approximate counts.

From Table 1 it is seen that whereas the strain obtained from the patient's blood before the ethylhydrocuprein treatment was started was readily killed by the pooled serum obtained during the administration of the drug, the strain obtained from the blood towards the end of the treatment and shortly before the death of the patient was completely insusceptible to any pneumococcal action of the same serum.

The effect of various concentrations of ethylhydrocuprein hydrochlorid in broth on the growth of the different strains of pneumococci obtained from this patient was also studied. In each test 0.1 c.c. of an eighteen-hour broth culture of the given strain was inoculated into 5 c.c. of the broth containing the ethylhydrocuprein, mixed and incubated for forty-eight hours. The degree of growth, if any, was judged macroscopically. Plates were also poured and smears examined after incubation.

The results given in Tables 1 and 2 show definitely that during the course of treatment in this case the pneumococci in the body became

gradually resistant to the action of the ethylhydrocuprein. The observations made in this case, together with the similar one made by us previously, not only demonstrate that this phenomenon of bacterial "fastness" may occur, but indicate that its occurrence is not infrequent.

TABLE 2.

Growth in Broth Containing Various Dilutions of Ethylhydrocuprein Hydrochlorid of Strains of Pneumococcus Isolated from Blood of Hospital No. 2825.

Designation of Strain	Isolated	Number of Colonies in 0.000001 C.c. of Culture Used in Tests
A	From blood on admission.....	85
B	From blood on second day of ethylhydrocuprein treatment.....	101
C	From blood on third day of ethylhydrocuprein treatment.....	162
D	From blood on fourth day of ethylhydrocuprein treatment (2 hrs. before death).....	150

Tube*	Dilution of Ethylhydrocuprein in Broth	Strain			
		A	B	C	D
1	1: 100,000	0	0	0	0
2	1: 200,000	0	0	0	0
3	1: 400,000	0	0	0	0
4	1: 600,000	0	0	0	Growth
5	1: 800,000	0	0	0	Growth
6	1: 1,000,000	0	0	Growth	Growth
7	1: 1,200,000	0	Growth	0	Growth
Control broth without ethylhydrocuprein		Growth	Growth	Growth	Growth

* Tubes incubated forty-eight hours at 37.5 C.

Bactericidal Action of Pericardial Fluid.

We have previously reported¹ that a pericardial exudate obtained post mortem from a patient who had received ethylhydrocuprein hydrochlorid by mouth for several days possessed bactericidal properties. In the present series of cases the same phenomenon was demonstrated in pericardial fluids obtained post mortem from four other

cases (Nos. 2800, 2845, 2919 and 3031). In each case the pericardial fluid was allowed to clot and the supernatant fluid was then pipetted off and used for bactericidal tests. Details of the tests of the pericardial fluid of two of these cases follow (Tables 3 and 4).

TABLE 3.

Test of Bactericidal Power of Blood Serum and Pericardial Fluid from Hospital No. 2800.

Tube Number	Blood Serum Obtained*	Number of Colonies of Pneumococci per 0.5 C.c. When Plated†		
		Immediately	After 1½ Hours Incubation	After 19½ Hours Incubation
1	Before ethylhydrocuprein.....	501	2,960	Confluent
2	12 hours after first dose.....	268	54	0
3	35 hours after first dose.....	421	121	0
4	Pericardial fluid obtained post mortem*...	301	345	1

* The pericardial fluid contained many pneumococci and it and the serum were heated at 56 C. for three-fourths hour before testing to destroy the contained pneumococci.

† Inoculation: 0.001 c.c. of a four-hour broth culture of *Pneumococcus* Type II.

TABLE 4.

Test of Bactericidal Power of Blood Serum and Pericardial Fluid from Hospital No. 2919.

Tube Number	Blood Serum Obtained	Number of Colonies of Pneumococci per 0.5 C.c. When Plated*		
		Immediately	After 1½ Hours Incubation	After 24 Hours Incubation
1	Before ethylhydrocuprein.....	1,200	2,400	Confluent
2	13 hours after first dose.....	1,200	Complete inhibition†	0
3	24 hours after first dose.....	800	Complete inhibition	84
4	69 hours after first dose.....	1,200	Complete inhibition	0
5	97 hours after first dose.....	1,000	Complete inhibition	0
6	Pericardial fluid obtained post mortem..	2,000	26

* Inoculation: 0.001 c.c. of a four-hour broth culture of *Pneumococcus* Type I.

† By complete inhibition of growth is meant no increase in the number of colonies

In Hosp. No. 2845 the pericardial fluid obtained post mortem showed pneumococcal action, although the blood serum obtained during the ethylhydrocuprein treatment showed only temporary inhibition of growth.

These observations show that when ethylhydrocuprein hydrochlorid is given by mouth according to the system of dosage used by us, it may pass into a serous sac (pericardial fluid) in amounts sufficient to exert a pneumococcal action.

Toxic Disturbances of Vision.

In one of the patient's of our former series¹ the administration of ethylhydrocuprein hydrochlorid led to the production of alarming and severe retinitis, from which, however, the patient recovered. In the present series eight patients complained of amblyopia, mild in three cases, more severe in five. On discontinuing the ethylhydrocuprein, vision was restored in all those who survived the pneumonia, and in the two who died the vision was improved after the discontinuance of the drug. Some details of these eight cases follow:

Hosp. No. 2940.—Housewife, aged 31; weight 74.2 kg.

Past History.—Unimportant.

Present Illness.—Chill, fever and pain in chest six days before admission.

Status on Admission.—Temperature 104.2 F.; pulse 136; respirations 44. Consolidation of left upper lobe; leukocyte count 41,400. Sputum tenacious, gray and contained *Pneumococcus* Type IV. Patient quite ill. Blood culture positive (four colonies per cubic centimeter).

Course and Treatment.—Ethylhydrocuprein hydrochlorid was started by mouth on the day of admission (seventh day of the disease) on a basis of 0.0269 gm. per kilogram of body weight for the first twenty-four hours (1×0.5 gm. + 10×0.15 gm.). During the second twenty-four hours 0.15 gm. was given every two hours. One hour after the second of these latter doses the patient complained of slight blurring of vision; later the vision seemed normal and the hearing became slightly impaired; about the time of the seventh dose (second twenty-four hours) the patient complained of "waves" before her eyes and continuous ringing in the ears and one and one-half hours after this dose she complained that everything was "blurred and indistinct." The ethylhydrocuprein was therefore discontinued and after about eight hours the patient was seen by Dr. W. W. Weeks. His report on her visual condition stated that both disks were pale, that there were numerous small areas of edema scattered throughout the fundi, and that the patient could at this time count fingers at a distance of 6

feet. The patient died two days after the ethylhydrocuprein was stopped. The total amount of ethylhydrocuprein given was 3.05 gm.

The bactericidal test of the serum of this case showed that pneumococcidal action for a stock strain of *Pneumococcus* Type II was present in the serum ten hours after the administration of the first dose of ethylhydrocuprein (plate poured immediately after inoculation showed 800 colonies; plate poured after one and one-half hours incubation, 400; and plate poured after twenty-four hours incubation was sterile). Pneumococcidal action was also present in serum obtained twenty-three hours after the initial dose. The pneumococcidal action was no longer present in serum obtained shortly before death (ethylhydrocuprein having been discontinued for two days).

Hosp. No. 2885.—Woman, cook, aged 42; weight 49 kg.

Past History.—Unimportant.

Present Illness.—Headache, pain in chest and vomiting two days before admission, followed next day by chill; little cough or expectoration.

Status on Admission.—Temperature 102.8 C.; pulse 96; respirations 32. Involvement of right upper lobe posteriorly; leukocyte count 28,000; sputum slightly tenacious, not hemorrhagic, contained *Pneumococcus* Type II.

Course and Treatment.—Ethylhydrocuprein hydrochlorid was started by mouth on the day following admission, on a basis of 0.03 gm. per kilogram of body weight for the first twenty-four hours (1×0.45 gm. + 7×0.15 gm.); thereafter 0.15 gm. every three hours. After a total amount of 1.95 gm. had been given, the patient complained that she could not see; the administration of ethylhydrocuprein was thereupon discontinued. The pupils at this time were not dilated. About one hour later the patient could distinguish the outline of persons. Examination of the eyegrounds revealed no marked abnormalities. On the following day the vision was much improved and the eyes were examined by Dr. W. W. Weeks, who reported that there was indistinctness of both disk margins, the fundi were pale, the veins were somewhat dilated and tortuous, the arteries were of normal size, the vision was 20/20 in both eyes, and the fields were normal. About this time the patient could distinguish colors and said she could see quite well. This patient received, in addition to the ethylhydrocuprein, 770 c.c. of antipneumococcus serum, Type II, intravenously, in divided doses. The temperature reached normal on the eighth day of the disease. Pneumococcidal power for a stock strain of *Pneumococcus* Type I was present in the serum of this case within twelve hours after the initial dose of ethylhydrocuprein.

Hosp. No. 2870.—Housewife, aged 46; weight 56.9 kg.

Past History.—Two previous attacks of pneumonia.

Present Illness.—Three days before admission, chill, nausea, pain in chest, cough, blood-tinged sputum.

Status on Admission.—Consolidation of left lower lobe; well marked friction rub over entire precordial region; area of cardiac dulness not enlarged. Tem-

perature 105.1; pulse 112; respirations 34. Sputum bright, rusty-red, mucopurulent and contained *Pneumococcus* Type II.

Course and Treatment.—Ethylhydrocuprein hydrochlorid was started by mouth the day after admission (fourth day of disease) on a basis of 0.0263 gm. per kilogram of body weight per twenty-four hours (1×0.45 gm. + 7×0.15 gm.; thereafter 0.15 gm. every two and a half hours). On the second day of treatment with ethylhydrocuprein the patient seemed slightly deaf and after 2.7 gm. of ethylhydrocuprein had been given, on being questioned, the patient said that she could not see. The pupils were widely dilated. The ethylhydrocuprein was thereupon discontinued. Three and a half hours later she said her sight was better, and in six hours after the last dose of ethylhydrocuprein she could distinguish objects. The next day her sight was considerably better and the pupils less dilated. The patient was seen by Dr. W. W. Weeks a few hours after the ethylhydrocuprein treatment had been discontinued. Dr. Weeks reported as follows: "Fingers can be seen 1 foot distant; visual fields moderately contracted; color not recognized; media clear; disks and fundi pale; veins engorged; arteries somewhat narrow." Dr. Weeks' report two days later was as follows: "Vision 20/40 + with both eyes; visual fields contracted, especially on nasal side; red or green cannot be distinguished; disks pale, especially on temporal side; fundi not pale; condition of vessels same as on previous examination." Her vision gradually improved and was normal when she left the hospital. Pneumococcal power for a stock strain of *Pneumococcus* Type I appeared in the blood serum of this patient within twenty-four hours of the administration of the first dose of ethylhydrocuprein.

Hosp. No. 2812.—Woman, aged 74; weight 58.5 kg.

Past History.—Unimportant.

Present Illness.—The patient said that she had not felt well for several days before admission. On the morning of the day before admission she felt very ill and had a temperature of 105 C., cough, blood-tinged sputum and pain in chest.

Status on Admission.—Temperature 104.1 F.; pulse 94; respirations 40. Involvement of the left lower lobe; emphysema present; sputum tenacious, rusty and yielded on passage through a mouse, *Pneumococcus mucosus* (Type III).

Course and Treatment.—Ethylhydrocuprein hydrochlorid was started by mouth on the day of admission, on a basis of 0.03 gm. per kilogram of body weight per twenty-four hours (1×0.6 gm. + 8×0.15 gm.; thereafter 0.15 gm. every two hours). After 0.9 gm. of the ethylhydrocuprein had been given the patient complained of hearing roaring noises, and after 3 gm. of ethylhydrocuprein she complained that she could not see. The ethylhydrocuprein was then discontinued. In six hours, however, she was able to count fingers and the next day her eyesight was much improved. Eight hours after the patient complained of loss of vision she was seen by Dr. W. W. Weeks, who reported that there was little change in the eyegrounds. The patient died on the fifth day after admission to the hospital.

Pneumococcidal action for a stock strain of *Pneumococcus* Type II appeared in this patient's serum six hours after the initial dose of ethylhydrocuprein.

Hosp. No. 3015.—Engineer, aged 47; weight 75.8 kg.

Past History.—Unimportant.

Present Illness.—Chill, vomiting, headache and pain in chest thirty-six hours before admission.

Status on Admission.—Temperature 104.6 F.; pulse 128; respirations 34. Consolidation of left lower lobe. Sputum obtained on admission was tenacious, frothy, rusty and contained *Pneumococcus mucosus* (Type III). Urine gave a definite precipitin reaction with antipneumococcus serum Type III.

Course and Treatment.—Ethylhydrocuprein hydrochlorid was started by mouth eleven and one-third hours after admission to the hospital on a basis of 0.034 gm. per kilogram of body weight per twenty-four hours (1×0.6 gm. + 10×0.2 gm.). After 2.4 gm. had been given the patient complained that he could not see distinctly and the ethylhydrocuprein was discontinued. At that time he could not see objects $1\frac{1}{2}$ feet from his eyes, but could distinguish direct light from darkness; the pupils were somewhat dilated and did not react to light; the veins were engorged and the eyegrounds somewhat pale. He had been questioned two and three-quarters hours previously as to whether he could see well, and replied that his vision was as good as normal; at that time he was partially deaf. Twelve hours after the complaint that vision was impaired, the patient could read a watch at 2 feet and a ward clock at about 20 feet, and said he could see well, but that objects were a little blurred. Later that day he was examined by Dr. W. W. Weeks, whose report is abstracted as follows: "Vision 20/40 + in both eyes; arteries injected; veins tortuous and engorged; left disk distinctly pale, especially the temporal half; visual fields restricted in both eyes, 10 degrees in right, and 20 degrees in left; color is recognized by left eye but not by right." Three days later Dr. Weeks reported as follows: "Pupils equal, contracted, reacted readily to light during accommodation and on convergence; vision without correction, O. D. 20/40 +, O. S. 20/210, with + 1.25 O. D. 20/20, with + 1.50 O. S. 20/20; concentric contraction for form, more in left eye; media clear; retina somewhat hazy near disks, so as to make disk margin a little indistinct; vessels normal except arteries of left eye which were somewhat smaller and more tortuous than those of right."

The temperature reached normal on the night of the day following admission to the hospital, but rose again thirty-six hours afterward and remained elevated for four days, although the pulse and respiration rates did not increase and the patient felt quite comfortable. The patient recovered.

The test of blood serum in this case showed pneumococcidal action for a stock strain of *Pneumococcus* Type II.

Hosp. No. 2972.—Man; complained of slight transient blurring of vision at times after the temperature had become normal; ethylhydrocuprein was discontinued (see Table 8 for details of the case).

Hosp. No. 2837.—Man; complained of transient deafness; transient dimness of vision occurred after the temperature had become normal; ethylhydrocuprein was discontinued (see details of case in Table 8); the patient had an abrasion of the cornea and there was some inflammation of the right cornea on the morning that he complained of dimness of vision.

Hosp. No. 2911.—Woman; complained of dimness of vision and slight partial deafness after she had received 2.95 gm. of ethylhydrocuprein hydrochlorid by mouth; both passed off after ethylhydrocuprein had been discontinued (for details of the case see Table 8).

In our entire series of seventy-five cases, nine, or 12 per cent. of the patients, showed some degree of amblyopia. Of all the patients treated with ethylhydrocuprein which are recorded in the literature, between 4 and 5 per cent. suffered from amblyopia, and in two of these the impairment of vision was more or less permanent (Oliver² and Lorant³). In these latter cases, however, the dosage of the drug seems to have been excessive.

Relation of Number of Pneumococci to Concentration of Ethylhydrocuprein in Bactericidal Tests.

In the technic employed in the bactericidal tests we have used a fairly constant and rather small number of pneumococci per cubic centimeter. It seemed advisable to determine whether or not the degree of bactericidal action would be the same when a larger number of pneumococci are employed. Specimens of broth containing a small amount of ethylhydrocuprein in solution were therefore inoculated separately with two different amounts of the same culture and incubated at 37.5 C. At frequent intervals plates were prepared and counts were made of the colonies which developed after incubation.

Experiment 1.—Fifty c.c. of bouillon containing ethylhydrocuprein hydrochlorid 1 to 1,000,000 were inoculated with 0.01 c.c. of a twenty-four hour culture of *Pneumococcus* Type II and a like amount of the same bouillon with 1 c.c. of the same culture. Similarly, two flasks containing 50 c.c. of broth each but without ethylhydrocuprein were inoculated with 0.01 c.c. and 1 c.c. of the culture, respectively, as controls. All four cultures were incubated at 37.5 C. and bacterial counts were made at frequent intervals by making suitable dilutions and pouring plates with 20 c.c. dextrose agar. The results of the experiment are given in Table 5.

2. Oliver, G. H.: Brit. Med. Jour., 1916, 1, 580.

3. Lorant, L.: Deutsch. med. Wchnschr., 1916, 42, 1355.

TABLE 5.

Number of Pneumococci in Relation to Action of Ethylhydrocuprein Hydrochlorid.

Incubation		Broth without Ethylhydrocuprein Inoculation 0.01 C.c.		Broth with Ethylhydrocuprein 1:1,000,000 Inoculation 0.01 C.c.		Broth without Ethylhydrocuprein Inoculation 1.0 C.c.		Broth with Ethylhydrocuprein 1:1,000,000 Inoculation 1.0 C.c.	
Hours	Minutes	No. of Viable Pneumococci per 0.5 C.c.	Log	No. of Viable Pneumococci per 0.5 C.c.	Log	No. of Viable Pneumococci per 0.5 C.c.	Log	No. of Viable Pneumococci per 0.5 C.c.	Log
0	0	28,000	4.447	19,050	4.379	2,455,000	6.390	2,450,000	6.389
2	0	19,300	4.285	11,450	4.05	2,870,000	6.457	1,685,000	6.226
4	0	9,750	3.989	475	2.676	7,050,000	6.848	1,005,000	6.002
5	30	11,450	4.058	37	1.568	22,500,000	7.352	625,000	5.795
7	0	11,850	4.073	3	0.047	26,500,000	7.423	370,000	5.568
9	0	34,500	4.537	0	0	49,500,000	7.694	210,000	5.322
11	35	625,000	5.795	0	0	67,500,000	7.829	100,000	5.000
25	..	49,500,000	7.694	0	0	55,500,000	7.744	240,000	5.380
48	51,000,000	7.707	45,000,000	7.653

FIGURE 1.

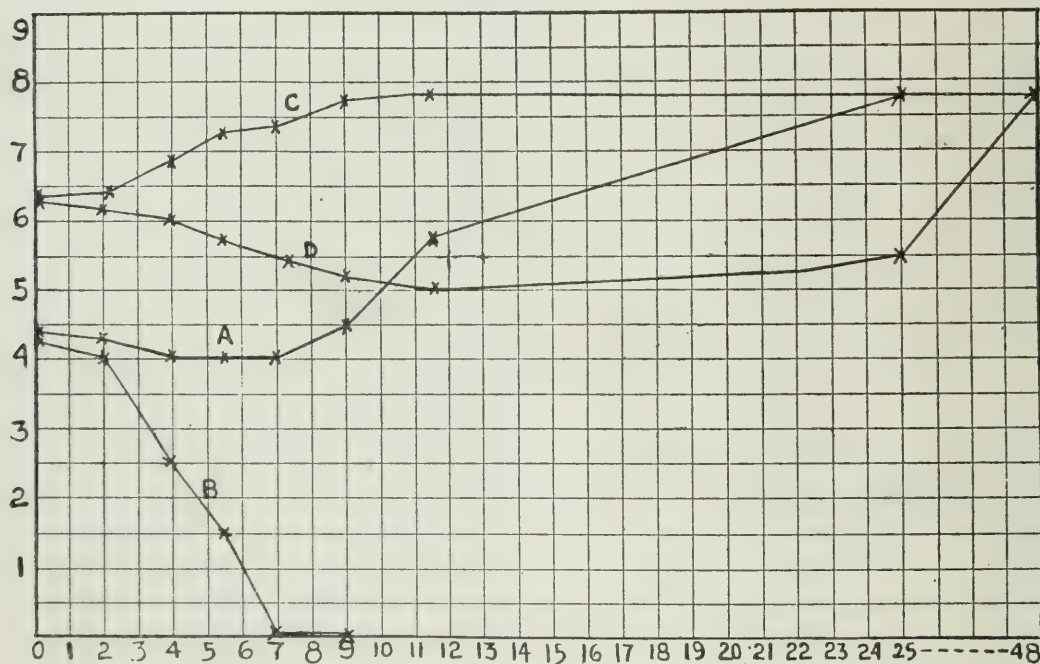


Diagram illustrating Experiment 1. Time in hours as abscissae at bottom, and at left logarithms of numbers of viable pneumococci per 0.5 c.c. as ordinates. A, Broth without ethylhydrocuprein; inoculation 0.01 c.c. B, Broth with ethylhydrocuprein (1:1,000,000); inoculation 0.01 c.c. C, Broth without ethylhydrocuprein; inoculation 1 c.c. D, Broth with ethylhydrocuprein (1:1,000,000); inoculation 1 c.c.

This experiment shows that while 19,050 pneumococci were all killed in nine hours in broth containing ethylhydrocuprein 1 to 1,000,000, 2,450,000 pneumococci were reduced to 100,000 in eleven hours and thirty-five minutes, but the surviving pneumococci were able to grow and in forty-eight hours multiplied almost to the same extent (45,000,000) as in the control broth culture. The logarithms of the bacterial count are shown in Table 5. The results of Experiment 1 are shown graphically by plotting curves (Fig. 1), employing the time in hours as abscissae and the logarithms of the numbers of viable pneumococci per 0.5 c.c. of culture as ordinates.

Penetration of Ethylhydrocuprein into Fibrinous Exudates.

In the course of our clinical and experimental observations on ethylhydrocuprein, we have received the impression that the drug does not readily penetrate the alveolar exudate. The concentration of ethylhydrocuprein in the blood of patients receiving the drug, according to the dosage most commonly used by us, is about 1 in 500,000, as judged by the pneumococcal action of the serum in the test tube. If ethylhydrocuprein in this concentration were to thoroughly penetrate the alveolar exudate, it should destroy the pneumococci present therein, and one might therefore anticipate a shortening of the duration of the disease. Since the duration of the disease is not shortened the following experiment was devised to study the power of ethylhydrocuprein to penetrate a fibrinous clot.

Experiment 2.—Fifty c.c. of normal rabbit blood were drawn into 1 c.c. of sterile 20 per cent. sodium citrate solution ($2\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot \text{H}_2\text{O}$) and centrifugalized. The supernatant plasma was drawn off and inoculated with a stock strain of *Pneumococcus* Type II (0.0001 c.c. of an eighteen-hour culture to 1 c.c. plasma) and 1.1 c.c. of sterile 9.5 per cent. calcium chlorid solution was then added and thoroughly mixed. The mixture was transferred to sterile cotton-plugged glass tubes 0.9 cm. in diameter, 2 c.c. being placed in each tube. A firm clot formed in each tube in about one to one and a half hours at room temperature. To one of the tubes 1.6 c.c. of 0.85 per cent. saline solution was then added, covering the clot, and, in a similar manner, to each of the remaining tubes there was added 1.6 c.c. of saline solution containing a given concentration of ethylhydrocuprein hydrochlorid. The tubes were kept at room temperature for one-half hour, then incubated for twenty hours at 37.5 C., and examined. The number of organisms used for the incubation had been so chosen that the control

tube which contained no ethylhydrocuprein showed small discrete colonies uniformly "peppering" the clot. In the tubes containing the more concentrated ethylhydrocuprein solutions no growth occurred in the upper portions of the clot. This was interpreted as due to the effect of the ethylhydrocuprein penetrating the clot in effective concentration to the depth of the zone of growth inhibition. The depth of the zone of inhibition bore a definite relationship to the concentration of the ethylhydrocuprein in the supernatant fluid. The zones of inhibition in the various tubes were measured and the results are shown in Table 6.

TABLE 6.

Inhibition by Ethylhydrocuprein of Growth of Pneumococci in Fibrinous Clots.

Dilution of Ethylhydrocuprein	Depth in Mm. of Zones of Inhibition of Growth	Dilution of Ethylhydrocuprein	Depth in Mm. of Zones of Inhibition of Growth
1: 100	10	1: 10,000	5.5
1: 500	9.5	1: 50,000	3.5
1: 1,000	9.0	1: 100,000	2.5
1: 5,000	6.0	1: 200,000	1.75
1: 10,000	5.5	1: 300,000	1.25
1: 50,000	3.5	1: 400,000	1.0
1: 100,000	2.5	1: 500,000	0.0
1: 500,000	0.0	1: 600,000	0.0
1: 1,000,000	0.0		
Control with saline	0.0	Control with saline	0.0

These experiments demonstrate that optochin, in the concentrations attainable with any degree of safety in the blood plasma of patients (about 1 in 500,000), possesses little power to penetrate a fibrinous clot. It is, therefore, possible that pneumococci in the interior of a pneumonic exudate may escape the action of ethylhydrocuprein, even though the drug be present in the blood stream in considerable concentration.

Clinical Observations.

In discussing the effect of ethylhydrocuprein on the clinical course of the disease, the patients treated during the two years will be considered as one series. As previously, they will be analyzed from the standpoint of (a) the duration of the disease, (b) the occurrence of "spread" or involvement of lobes hitherto unaffected, (c) the effect of treatment on pneumococcemia and (d) mortality with reference

to the immunologic classification of the infecting strain of pneumococcus.

(a). *Effect on Duration of Disease.*—Using the occurrence of a rectal temperature below 100 F. as a criterion for the termination of the acute attack, an arbitrary but serviceable standard, the average duration of the disease in all the recovered patients was eight days. No marked shortening, therefore, of the course of the disease can be said to have occurred in our series.

(b). *Occurrence of "Spread" of Pulmonary Lesion.*—Of the 75 cases, 20, or 26.6 per cent., showed a "spread" during treatment. In 14 of these 20 cases the serum of the patient possessed bactericidal power at the time the spread took place; this may be taken as presumptive evidence that the drug, although circulating in the blood in amounts sufficient to destroy pneumococci in vitro, does not pass into the alveolar spaces or exudate in amounts sufficient to destroy the bacteria therein or inhibit their growth to any marked extent.

(c). *Pneumococcemia.*—In 23, or 30.6 per cent. of the 75 cases, viable pneumococci were present in the circulating blood at some time during the course of the disease. In many instances there were only a few pneumococci present, whereas in others there was a comparatively heavy blood infection. In 19 of the 23 cases the blood culture was positive before ethylhydrocuprein treatment was begun; in 4 of these the blood culture became negative during treatment with ethylhydrocuprein alone; 9 of the cases showed a progressively increasing number of micro-organisms while under treatment; and the remaining 6 died without subsequent blood cultures having been made. When in addition to ethylhydrocuprein immune serum was used in treatment, the number of pneumococci in the circulating blood was always reduced after the first administration of serum, but in one instance the number of micro-organisms later increased even though the patient received both ethylhydrocuprein and serum. In 4 instances the blood culture was negative at the time the ethylhydrocuprein treatment was instituted, but became positive later. From these results it may be stated that no marked beneficial effect of ethylhydrocuprein treatment was observed on the pneumococcemia of lobar pneumonia.

(d). *Mortality.*—Of the 75 patients treated, 28 died—a mortality rate of 37.3 per cent. Some of the patients were inadequately treated.

judging from the production of pneumococidal power in the serum. Moreover, in several cases treatment was instituted less than twenty-four hours before death and at a time when the patients were critically ill; the duration of administration of the drug in these cases was, therefore, too short to be of any value, considering the time required for its absorption and action. If all the patients who are known to have been inadequately treated, regardless of whether they died or recovered, be excluded, and also those cases in which treatment was instituted within twenty-four hours of death, or in which crisis occurred before the serum acquired bactericidal power, there would remain 51 cases, of whom 16 patients died—a mortality of 31.3 per cent. Of the 24 cases excluded from consideration 12 patients died and 12 recovered. The mortality rate in the 51 adequately treated patients, 31.3 per cent., does not show any considerable therapeutic effect from the use of the drug.

It should be stated that the series probably represents a group of very severe cases, for it is composed for the most part of cases due to infection with pneumococci of Types II and III, which we know are the types responsible for the highest mortality rates. When the expected mortality in each of these groups (untreated) is compared with that actually encountered in the patients treated with ethylhydrocuprein, we find some reduction of mortality rate in the cases infected with pneumococci belonging to Group III, and no reduction in the cases due to pneumococci of Groups II and IV. This will be seen from a study of Table 7, in which the cases are arranged according to the type of infecting pneumococcus and in which the mortality obtained in the various groups in our series is contrasted with that obtained in a large series of patients not treated specifically and observed in different clinics.¹ One is forced to conclude from these figures that treatment with ethylhydrocuprein hydrochlorid failed to cause any reduction in the general mortality rate.

Of the 75 patients treated with ethylhydrocuprein, 14 in which the disease was caused by pneumococci belonging to Group II were treated, in addition, with the type homologous antipneumococcus serum. This agent was used in the form of the whole serum intravenously or the concentrated "extract"⁴ of the serum subcutaneously

4. Chickering, H. T.: Jour. Exper. Med., 1915, 22, 248.

and intravenously. Of these 14 patients 10 recovered and 4 died, giving a mortality rate of 28.5 per cent., so that no reduction in the mortality rate was observed in cases of lobar pneumonia due to *Pneumococcus* Type II as a result of treatment with ethylhydrocuprein and antipneumococcus serum. The number of patients treated with serum and ethylhydrocuprein is too small, however, to permit of final conclusions on this point.

TABLE 7.

Mortality Among Patients Treated with Ethylhydrocuprein Hydrochlorid Compared with That of Patients Not Specifically Treated.

Pneumococcus Type	Number of Patients Treated with Ethylhydrocuprein	Recovered	Died	Mortality Rate Per cent.	Average Mortality Rate Per Cent. in Patients Not Specifically Treated
II	27	19	8	29.6	28
III	17	10	7	41.1	56
IV	6	5	1	16.6	16
Unclassified	1	1	0	0	..
	51	35	16	31.3	About 26

DISCUSSION.

The experimental studies which we have here discussed and those previously reported show that ethylhydrocuprein hydrochlorid fulfills at least some of the requirements of a chemotherapeutic agent in lobar pneumonia. Even in high dilutions it kills the pneumococcus in the presence of body fluids. It is capable of being absorbed from the gastro-intestinal tract, and when injected into the muscles (Case 2947, Table 8) may pass into the blood stream. Moreover, when a sufficient amount is administered by mouth, represented by 0.024 to 0.028 gm. per kilogram of body weight per twenty-four hours, the blood serum almost always becomes pneumococcidal in vitro, and furthermore, when such a condition obtains in the blood, the pericardial fluid also becomes pneumococcidal. The amount of the drug which it is necessary to administer in order to achieve this result, however, cannot always be given with safety to the patient, for in one instance in our

series of cases total blindness lasting six days resulted, and in eight other instances there occurred visual symptoms of sufficient gravity to make the discontinuance of the drug necessary. A study of Table 8 would seem to suggest that where there is a comparatively heavy septicemia (for example, 100 colonies per 1 c.c. or over) a dosage of ethylhydrocuprein represented by 0.024 to 0.026 gm. per kilogram of body weight per twenty-four hours may be insufficient to produce pneumococcidal actions in the blood serum (Case Nos. 2845 and 2892). It is possible that in such instances the drug may be fixed by the circulating pneumococci. In one case without pneumococcemia (No. 2822) which received 0.024 gm. of the hydrochlorid of the drug per kilogram of body weight per twenty-four hours in suitably divided doses, pneumococcidal action failed to appear in the serum.

When the cases in our series are analyzed from the standpoint of the effect of the drug on the duration of the disease, on the occurrence of "spread" of the lesion to previously uninvolved lobes of the lung, on the pneumococcemia and on the mortality rate, the results do not afford much support for the routine use of this drug in the treatment of acute lobar pneumonia.

It seems to us that the main reason why ethylhydrocuprein has not produced more striking results in the treatment of lobar pneumonia is because the toxicity of the drug is such as to keep the limits of dosage below the limits of effectiveness. If larger doses could be safely employed, it would be possible greatly to increase the amount of ethylhydrocuprein circulating in the blood. This would in turn undoubtedly increase the rate and degree of the resulting pneumococcidal action in the blood and might conceivably lead to a greater penetration of the drug into the consolidated portions of the lung. Our tests show that ethylhydrocuprein may be administered to patients in amounts sufficient to cause the serum to acquire pneumococcidal power. With the dosage that may be safely employed, however, the serum exhibits its bactericidal activity only at a slow rate, considerable time being required. Because of this fact the pneumococci in the body may be exposed for considerable time to concentrations of the drug insufficient to cause their destruction, particularly when they are protected by the comparative impenetrability of a solid pneumonic exudate. Under

such circumstances, as we have shown, pneumococci may become resistant or "fast" to the action of the drug.

As our experiments indicate, there seems to be some relationship between the number of pneumococci and the concentration of ethylhydrocuprein which is required to completely kill them. Thus, in test tube experiments it has been shown that whereas a given amount of the drug in solution is sufficient to destroy a given number of pneumococci per unit volume, the same amount of drug is not able to destroy 100 times this number in the same volume. Inasmuch as we cannot estimate in any human case the number of pneumococci which it is necessary to destroy, it is quite conceivable that much larger amounts of the drug than we have found it safe to administer may be necessary to produce the required concentration in the body fluids.

Finally, it is probable that in the concentration which may safely be attained in the blood stream of the patient (about 1 in 500,000), the drug does not penetrate the alveolar exudate to any marked degree and therefore cannot kill the pneumococci there present.

Our conception of the present status of ethylhydrocuprein therapy in lobar pneumonia is, then, that while much of the experimental evidence is favorable, the clinical results that have been obtained are scarcely sufficient to warrant the routine administration of a drug the use of which may result in damage to vision. Probably the drug would be efficient if it could be given in larger amounts. As a "lead" in chemotherapy the drug is of great value; synthetic study of the quinin alkaloids should be made for a compound possessing greater pneumococidal power in the presence of body fluids, greater velocity of action on pneumococci and less toxicity. Such a compound should, in addition, possess the power of rapid and easy penetration into the alveolar exudate. With such a drug at our disposal we might expect in lobar pneumonia something approaching a *therapia sterilisans magna*.

TABLE 8.—S

Hospital Case Number	Age	Weight, Kg.	Type of Infecting Pneumococcus	Day of Disease When Ethylhydrocuprein Treatment Was Begun	Lung Involvement on Admission	Blood Culture Before Treatment	Method of Dosage of Ethylhydrocuprein in Periods of 24 Hrs.* Gm. (First two periods)	Amount of Ethylhydrocuprein per Kilogram of Body Weight per 24 Hrs. Gm.	Total Amount of Ethylhydrocuprein in Grams
2753	27	55.8	II	3	L. L.	Sterile	0.45+7×0.15; 10×0.15	0.0269	5.7
2754	13	39.6	II	8	L. U. L. L.	0.3+7×0.1; 10×0.1	0.0252	2.3
2783	48	63.3	III	7	R. U. R. L.	Positive; 325 col. per 1.0 c.c.	0.45+0.25+0.3; 2×0.15; base	1.3
2786	34	42.7	II	8	R. U.	Sterile	0.3+0.2+7×0.10; 10×0.1; base	0.028	2.2
2797	67	78.2	III	2	R. U.	Sterile	0.6+2×0.25+6 ×0.15; 11×0.2	0.023	10.8
2800	42	78.9	III	4	L. L.	Sterile	0.6+0.3+8×0.2; 1.65 in 2nd 24 hrs.	0.03	4.1
2812	74	58.5	III	2	L. L.	Sterile	0.6+8×0.15; 12×0.15	0.03	3.0
2822	13	37.2	II	6	L. L.	Sterile	0.2+7×0.1; 9×0.1; 10×0.1	0.0241	3.6

* Given by mouth unless otherwise stated; hydrochlorid used unless otherwise stated

Duration of Disease in Days	Toxic Symptoms Referable to Ethylhydrocuprein	Complications	Occurrence of "Spread" During Treatment	Result	Remarks
7	None	None	None	Recovery	
10	None	Pericarditis	None	Death	
7	None	Pericarditis	None	Death	Blood culture 8 hours after treatment showed innumerable colonies per 0.5 cc. blood
9	None	None	None	Recovery	
7	None	None	Spread to right lower	Death	
6	None	None	None	Death	Blood culture 24 hours after treatment was begun showed 23 colonies per 1.0 cc. blood; pericardial fluid showed bactericidal action
6	Temporary blindness, deafness and tinnitus	None	None	Death	Ethylhydrocuprein discontinued on account of eye symptoms
10	None	None	None	Recovery	

Hospital Case Number	Age	Weight, Kg.	Type of Infecting Pneumococcus	Day of Disease When Ethylhydrocuprein Treatment Was Begun	Lung involvement on Admission	Blood Culture Before Treatment	Method of Dosage of Ethylhydrocuprein in Periods of 24 Hrs * Gm. (First two periods)	Amount of Ethylhydrocuprein per Kilogram of Body Weight per 24 Hrs. Gm.	Total Amount of Ethylhydrocuprein in Grams
2762	23	70.0	III	3	R. L.	Sterile	$0.45+8\times 0.15;$ 3×0.5 per rectum	0.0235	3.1
2825	50	69.0	II	2	L. L.	Positive; 3 col. per 1 c.c.	$0.6+8\times 0.15;$ 12×0.15	0.026	6.5
2827	29	52.6	II	3	L. L. L. U.	Positive; 2,000 col. per 1 c.c.	$0.45+2\times 0.15$	0.0285	0.7
2831	35	57.0	II	5	L. L.	Sterile	$0.45+7\times 0.15;$ 10×0.15	0.0263	1.6
2834	28	58.4	II	5	L. L.	Sterile; 16 hrs. after ethylhydrocuprein positive—12 col. per 1 c.c.	$0.5+0.2+6\times 0.15;$ $9\times 0.15; 10\times 0.15$	0.0274	9.0
2837	48	46.0	III	2	R. L.	Sterile	$0.45+7\times 0.15;$ 7×0.2	0.0326	2.9

* Given by mouth unless otherwise stated; hydrochlorid used unless otherwise stated

	Duration of Disease in Days	Toxic Symptoms Referable to Ethylhydrocuprein	Complication	Occurrence of "Spread" During Treatment	Result	Remarks
s.	5	Persistent vomiting; partial deafness	None	None	Recovery	
s.	5	None	None	None	Death	Progressive increase in septicemia during treatment. "Fast" strains recovered from blood; anti-pneumococcus serum 85 c.c. intravenously on last day
	4	None	None	None	Death	
	6	None	None	None	Recovery	75 c.c. antipneumococcus serum intravenously
	11	None	None	Spread to right lower	Death	Active maniacal delirium. Blood culture positive 16 hours after ethylhydrocuprein was started; 1,015 c.c. antipneumococcus serum intravenously
n.	4	Transient deafness; slight dimness of vision after temp had become normal	None	None	Recovery	Ethylhydrocuprein discontinued after temp. became normal on account of eye symptoms. Eyegrounds apparently normal on ophthalmoscopic examination at time patient complained of dimness of vision

Hospital Case Number	Age	Weight, Kg.	Type of Infecting Pneumococcus	Day of Disease When Ethylhydrocuprein Treatment Was Begun	Lung Involvement on Admission	Blood Culture Before Treatment	Method of Dosage of Ethylhydrocuprein in Periods of 24 Hrs.* Gm. (First two periods)	Amount of Ethylhydrocuprein per Kilogram of Body Weight per 24 Hrs. Gm.	Total Amount of Ethylhydrocuprein in Grams
2838	45	48.8	III	4	R. L.	Sterile	0.5+5×0.2; 8×0.2; 6×0.25	0.0307	7.6
2845	46	40.4	II	5	R. U.	Positive; 1,000 col. per 1 c.c.	0.5+4×0.25; 6×0.25	0.0371	2.0
2849	42	94.4	III	4	R. U.	Positive; 1 col. in 3 c.c.	0.5+4×0.25; 6×0.25	0.0158	2.0
2865	23	60.4	II	4	L. U.	Sterile	0.45+7×0.15; 10×0.15	0.0248	5.2
2869	31	66.6	II	2	R. L.	Positive; 400 col. per 1 c.c.	0.45+7×0.15; 10×0.15	0.0225	5.3
2870	46	56.8	II	4	L. L.	Sterile	0.45+7×0.15; 8×0.15	0.0263	2.7
2879	26	79.0	II	3	L. L.	Positive	0.5+9×0.15; 10×0.15	0.0234	3.4
2885	42	49.0	II	3	R. U.	Sterile	0.45+7×0.15	0.0300	1.9

* Given by mouth unless otherwise stated; hydrochlorid used unless otherwise stated

Case No.	Duration of Disease in Days	Toxic Symptoms Referable to Ethylhydrocuprein	Complications	Occurrence of "Spread" During Treatment	Result	Remarks
Chin Mrs.	9	None	None	None	Recovery	
. A.; Library tition ly	7	None	None	None	Death	Pericardial fluid showed bactericidal action; 300 c.c. antipneumococcus serum intravenously
. A.	5	None	None	None	Death	Blood culture before death, 1,000 colonies per 1.0 c.c. blood
Chin Mrs.	7	None	None	None	Recovery	
. A.	6	None	None	Spread to right upper	Death	Blood culture constantly positive; 10,000 colonies per 1.0 cc. just before death; 580 c.c. antipneumococcus serum intravenously
Chin Mrs.	9	Transient amblyopia and deafness	None	None	Recovery	Ethylhydrocuprein discontinued on onset of amblyopia
. A.; tem- y in- tion	5	None	None	None	Recovery	Blood culture became negative during treatment; 440 c.c. antipneumococcus serum intravenously
Chin Mrs.	8	Transient amaurosis and tinnitus	None	None	Recovery	Ethylhydrocuprein discontinued on account of eye symptoms; 770 c.c. antipneumococcus serum intravenously; delayed resolution

Hospital Case Number	Age	Weight, Kg.	Type of Infecting Pneumococcus	Day of Disease When Ethylhydrocuprein Treatment Was Begun	Lung Involvement on Admission	Blood Culture Before Treatment	Method of Dosage of Ethylhydrocuprein in Periods of 24 Hrs.* Gm. (First two periods)	Amount of Ethylhydrocuprein per Kilogram of Body Weight per 24 Hrs. Gm.	Total Amount of Ethylhydrocuprein in Grams
2886	21	59.8	II	4	L. U.	Sterile	0.45+7×0.15; 11×0.15	0.0250	5.25
2892	41	59.0	II	5	R. U.	Positive; 100 col. per 1 c.c.	0.45+7×0.15; 10×0.15	0.0254	2.25
2890	28	35.5	II	4	R. L.	Positive; 3 col. per 1 c.c.	0.45+8×0.15; 10×0.15	0.0464	5.4
2897	34	59.0	II	4	L. U.	Sterile	0.45+7×0.15; 10×0.15	0.0254	6.0
2898	32	53.2	III	5	L. L.	Positive; 120 col. per 1 c.c.	0.45+7×0.15; 10×0.15	0.0283	3.75
2911	50	74.2	III	3	R. U.	Sterile	0.45+7×0.15+1 ×0.1; at rate of 10×0.15 thereafter	0.021	2.95
2919	35	49.8	III	4	L. L.	Sterile	0.45+7×0.15; 10×0.15	0.03	6.30

* Given by mouth unless otherwise stated; hydrochlorid used unless otherwise stated

ed.

after initial dose of Ethylhydrocuprein	Duration of Disease in Days	Toxic Symptoms Referable to Ethylhydrocuprein	Complications	Occurrence of "Spread" During Treatment	Result	Remarks
thin rs.	8	Slight deafness	None	None	Recovery	
. A.; tem- y in- ion	7	None	None	Spread to right lower	Death	Blood culture before death, 600 colonies per 1.0 c.c. blood
thin rs.	8	None	Pneumococcal meningitis	Spread to left lower	Death	Blood culture 18 hours after ethylhydrocuprein was commenced showed 14 colonies per 1 c.c.; 1,300 c.c. serum intravenously; 80 c.c. serum intraspinaly; 30 c.c. concentrated serum subcutaneously
thin rs.	8	None	None	None	Recovery	380 c.c. serum intravenously; 74 c.c. concentrated serum subcutaneously
thin rs.	8	None	None	Spread to right lower	Death	
plete tion	8	Temporary blurring of vision and temporary partial deafness	None	None	Recovery	Ethylhydrocuprein discontinued on account of eye symptoms
thin rs.	9	None	None	Spread	Death	Pericardial fluid showed bactericidal action; blood culture sterile post mortem

Hospital Case Number	Age	Weight, Kg.	Type of Infecting Pneumococcus	Day of Disease When Ethylhydrocuprein Treatment Was Begun	Lung Involvement on Admission	Blood Culture Before Treatment	Methods of Dosage of Ethylhydrocuprein in Periods of 24 Hrs. * Gm. (First two periods)	Amount of Ethylhydrocuprein per Kilogram of Body Weight per 24 Hrs. Gm.	Total Amount of Ethylhydrocuprein
2922	30	58.0	II	4	L. L.	Sterile	0.45+7×0.15; 10×0.15	0.0258	11.
2926	19	46.0	II	5	R. U.	Sterile	0.45+5×0.15; 7×0.15	0.026	3.
2927	22	56.4	III	3	R. L.	Sterile	0.45+7×0.15; 8×0.15	0.0265	4.
2940	31	74.2	IV	7	L. U.	Positive; 4 col. per 1 c.c.	0.5+10×0.15; 12×0.15	0.0269	3.
2943	22	44.4	II	5	R. L.	Sterile	0.4+8×0.1; 12×0.1	0.027	4.
2946	28	62.6	II	4	R. U.	Sterile	0.5+8×0.15; 12×0.15	0.0271	12.
2947	51	70.4	III	6	R. L.	Positive	0.7 intramuscularly + single dose of 1.8 gm. intramuscularly	0.0355	9.
2973	40	50.2	III	3	R. U.	Sterile	0.45+7×0.15; 10×0.15	0.0375	3.
2972	19	55.6	II	1	L. L.	Sterile	0.45+7×0.15; 10×0.15	0.0269	6.

* Given by mouth unless otherwise stated; hydrochlorid used unless otherwise stated

Duration of Disease in Days	Toxic Symptoms Referable to Ethylhydrocuprein	Complications	Occurrence of "Spread" During Treatment	Result	Remarks
14	None	None	Spread to left upper	Recovery	197 c.c. concentrated serum subcutaneously
8	Slight deafness	None	None	Recovery	
6	Temporary deafness	None	Spread to left upper	Recovery	
11	Dimness of vision and tinnitus	None	Death	Ethylhydrocuprein discontinued on account of eye symptoms; eye symptoms disappeared
10	None	None	Spread to right upper	Recovery	
12	None	None	Spread to right lower	Death	
?	None	Empyema	Spread to left lower	Operation; death 60 days after admission	Blood culture became negative; intermission of 90½ hours in ethylhydrocuprein treatment after 2.5 gm. had been given
6	Temporary deafness	None	None	Recovery	
6	Slight transient blurring of vision after temp. had become normal	None	None	Recovery	

Hospital Case Number	Age	Weight, Kg.	Type of Infecting Pneumococcus	Day of Disease When Ethylhydrocuprein Treatment Was Begun	Lung Involvement on Admission	Blood Culture Before Treatment	Method of Dosage of Ethylhydrocuprein in Periods of 24 Hrs.* Gm. (First two periods)	Amount of Ethylhydrocuprein per Kilogram of Body Weight per 24 Hrs. Gm.
2991	49	71.2	II	2	R. L.	Sterile	0.45+9×0.15; 6×0.3	0.025
3006	35	46.8	II	3	R. L.	Sterile	0.45+7×0.15; 10×0.15	0.032
3015	47	75.8	III	3	L. L.	Sterile	0.6+10×0.2	0.034
2868	20	57.0	II	5	R. L.	Sterile	0.45+7×0.15	0.0263
3031	38	57.0	II	3	L. L.	Positive; 80 col. per 1 c.c.	0.45+8×0.15; 9×0.15; 10×0.15	0.028

* Given by mouth unless otherwise stated; hydrochlorid used unless otherwise stated.

CONCLUSIONS.

1 Ethylhydrocuprein (optochin) base is absorbed with difficulty into the blood stream from the gastro-intestinal tract; the hydrochlorid of the drug is readily absorbed.

2. During treatment with ethylhydrocuprein pneumococci in the human body can gradually become "fast" or resistant to its action.

3. The pericardial fluid obtained post mortem from patients treated with ethylhydrocuprein hydrochlorid showed pneumococcidal power.

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after Initial Dose of Ethylhydrocuprein	Duration of Disease in Days	Toxic Symptoms Referable to Ethylhydrocuprein	Complications	Occurrence of "Spread" During Treatment	Result	Remarks
not died	9	Temporary deafness	Delayed resolution	None	Recovery	
hin Mrs.	8	None	None	Spread to left lower	Death	
hin Mrs.	?	Temporary blindness	None	None	Recovery	Ethylhydrocuprein discontinued on account of visual symptoms
not died	12	None	None	None	Recovery	Ethylhydrocuprein discontinued because it was thought that patient had had crisis
hin Mrs.	8	None	None	None	Death	Strain obtained just after death was not "fast" to ethylhydrocuprein; pericardial fluid obtained post mortem showed pneumococcal action for Type I pneumococcus

4. The serum of one patient who received a very large dose of ethylhydrocuprein hydrochlorid intramuscularly showed pneumococcal power (Case 2947, Table 8).

5. Among seventy-five patients treated with ethylhydrocuprein there were nine who showed some degree of amblyopia (12 per cent.); this was mild in three cases and more severe in six. In all those who recovered from the pneumonia, the eye symptoms disappeared completely after the administration of the drug had been discontinued.

6. The systematic use of ethylhydrocuprein (optochin) in the treatment of seventy-five cases of acute lobar pneumonia due to pneumococci did not lead to any noteworthy therapeutic benefit. The failure of the ethylhydrocuprein treatment to influence favorably the course of the disease is probably due to the following:

(a). It is impossible to administer a sufficient amount of the drug to produce an effective concentration in the blood stream without at the same time exposing the patient to the danger of toxic action.

(b). The rate of the pneumococidal action of ethylhydrocuprein is too slow in the concentrations which may be attained in the blood stream of the patient with any degree of safety; pneumococci, therefore, may gain access to the circulating blood at a greater rate than they are destroyed therein, even though the serum show pneumococidal action.

(c). In the concentrations which are safely attained in the body fluids the drug probably penetrates but poorly into the alveolar exudate.

7. The routine use of ethylhydrocuprein in the treatment of acute lobar pneumonia cannot be recommended.

HYDROGEN ION CONCENTRATIONS OF VARIOUS INDICATOR END-POINTS IN DILUTE SODIUM HYPOCHLORITE SOLUTIONS.*

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In the preparation of Dakin's hypochlorite solution it has been customary to adjust the reaction by the use of powdered phenolphthalein. After precipitating the calcium of the bleaching powder with Na_2CO_3 , Dakin added boric acid to the strongly alkaline hypochlorite solution until it no longer colored powdered phenolphthalein. Dayfresne modified this technique by using sodium bicarbonate in place of boric acid, but still used powdered phenolphthalein as the test for the reaction of the solution. Such a solution has frequently been described somewhat loosely as a "neutral" solution of hypochlorite, but it is not neutral. Because of the bleaching action of the hypochlorite, the end-points of indicators in hypochlorite solution are quite different from the end-points of the same indicators in ordinary solutions. A much greater alkalinity is required to give a color with powdered phenolphthalein in the presence of hypochlorite than in its absence. Moreover, the degree of alkalinity required in a hypochlorite solution to give a color with powdered phenolphthalein is much greater than that required to give a flash of color with alcoholic phenolphthalein. As alkalinity in these solutions increases their stability, their irritating effects upon the skin and tissues, and their solvent action on necrotic tissue (Dakin; Dakin and Dunham; Carrel and Dehelly; Fiessinger and coworkers; Taylor and Austin), it seemed desirable to define as accurately as possible the hydrogen

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ion concentrations actually indicated by these two end-points.¹ We have used powdered phenolphthalein and 1 per cent alcoholic solution of phenolphthalein. To these we have added 0.1 per cent alcoholic solution of *o*-cresolphthalein, which in hypochlorite solutions has an end-point between those of powdered and alcoholic phenolphthalein. *o*-Cresolphthalein, first made by Fraude (1880), has been studied and recommended by Clark and Lubs as a more brilliant indicator than phenolphthalein over about the same range.

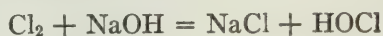
The hypochlorite solutions have been prepared by passing chlorine gas through solutions, either of sodium hydroxide or of sodium carbonate, of known concentration. The chemical reactions under these conditions may be represented as follows:

With sodium hydroxide:

(1) With equivalent molecular proportions of sodium hydroxide and chlorine:



(2) With excess of chlorine:



With sodium carbonate:



Actually at any given moment in the reaction there exist proportions of Na_2CO_3 , NaHCO_3 , NaOCl , and HOCl determined by the alkalinity and degree of chlorination. Starting with either Na_2CO_3 or NaOH , as the chlorine is introduced, the alkalinity of the solution is diminished and the total hypochlorite ($\text{NaOCl} + \text{HOCl}$) increased. With any given solution of either type therefore the change in the thiosulfate titration as the chlorine is introduced is an approximate gauge of the change in C .

EXPERIMENTAL.

A series of experiments was made in which, starting with a known solution of sodium hydroxide, chlorine gas was introduced to the end-

¹ Clowes recognized the variations that occurred in hypochlorite solutions, prepared with powdered phenolphthalein as the sole test of reaction and suggested a further control by titration of the alkali content.

point of one or more of the three indicators, and at these points samples of the solution were titrated with thiosulfate. The results are given in Table I. It is evident that there is a fairly constant relation between the original concentration of the sodium hydroxide and the thiosulfate titrations at the end-points to powdered phenolphthalein and to *o*-cresolphthalein. The end-point to *o*-cresolphthalein is a little less alkaline than that to powdered phenolphthalein. The titration at the end-point to alcoholic phenolphthalein is much less constant. This is due to two factors: first, the difficulty of reading the evanescent flash of color; second, and we believe more important,

TABLE I.
Sodium Hydroxide and Chlorine.

Original solution NaOH per 100 liters.	0.1 N thiosulfate per 10 cc. at end-point to		
	Powdered phenolphthalein.	<i>o</i> -Cresolphthalein.	Alcoholic solution of phenolphthalein.
<i>gm. mols.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>
28.3		28.5	33.1
27.8	27.0		35.3
27.8			32.8
27.7	27.3	30.1	37.2
27.7	27.4	29.4	
27.5			31.6
26.9	26.7		38.2
12.8		13.1	
12.8		12.9	

the formation at this C_H of other acids, HClO_2 , HClO_3 , HClO_4 , as well as HClO , the three former being produced in varying amounts depending upon the precise conditions of the experiment and thus altering considerably the relation between the C_H and the thiosulfate titration.

Similar experiments starting with a known sodium carbonate solution (Table II) showed much greater variation in the relation between the C_H and the thiosulfate titration at all three end-points. This is presumably due to the partial escape of CO_2 liberated in the reaction. As the extent to which this escape of CO_2 takes place varies somewhat depending upon the rate at which the chlorine is introduced, the fineness of the bubbles in which it is introduced, the thorough-

ness of the stirring, and the temperature of the solution, this variation between C_H and thiosulfate titration is readily understood.

In addition to the thiosulfate titrations, analyses were made in these solutions of the total chlorides, of the alkalies, and of the carbonate contents, so that the approximate composition of the solutions could be calculated. The ratios of $\frac{HOCl}{NaOCl}$ at the various indicator end-points as determined in the sodium hydroxide experiments were made use of in the calculation of the composition of the carbonate solutions at these same end-points. Finally, from the

TABLE II.

Sodium Carbonate and Chlorine.

Original solution Na_2CO_3 per 100 liters.	0.1 N thiosulfate for 10 cc. sample at end-point to		
	Powdered phenolphthalein.	<i>o</i> -Cresolphthalein.	Alcoholic solution of phenolphthalein.
<i>gm. mols.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>
28.8	17.2		35.7
28.6			34.3
27.4	12.5	27.0	
27.3	19.8		35.0
27.1	13.8	27.7	33.6
27.0	14.9		35.0
12.9	9.8	13.2	16.9
12.9	8.9	13.6	17.4

calculated $\frac{NaHCO_3}{Na_2CO_3}$ ratios of the carbonate solutions at these end-points the C_H values were calculated by McCoy's figures.

$$C_H = \frac{NaHCO_3}{Na_2CO_3} \times \frac{6.0 \times 10^{-11}}{0.8}$$

All hydrogen ion concentrations will be expressed by Sørensen's symbol pH; *i.e.*, $pH = \log \frac{1}{C_H}$

Methods.

End-Points.—The introduction of the chlorine gas into the alkaline solutions was stopped at frequent intervals and 3 to 4 cc. portions of

the hypochlorite solutions were removed and placed in small test-tubes to be tested as follows:

Powdered Phenolphthalein.—A few mg. of the powder were placed upon the solution and the tube was shaken. The first test failing to develop the red or pink color was taken as the end-point.

o-Cresolphthalein.—Several drops (8 to 10) of 0.1 per cent alcoholic solution of *o*-cresolphthalein were squirted into the solution from a medicine dropper. A definite flash of purplish red color was considered an indication of greater alkalinity than the end-point. After the end-point is reached a dirty grayish color may appear upon addition of the indicator, but this was disregarded.

Alcoholic Phenolphthalein.—A 1 per cent alcoholic solution was used in exactly the same way as the 0.1 per cent alcoholic solution of *o*-cresolphthalein. The flash of color is red, not purplish, in this case.

Analyses.—The alkali solutions before chlorination were titrated with decinormal acid. The chlorinated solutions were titrated for total hypochlorite (NaOCl and HOCl), with 0.1 *N* sodium thiosulfate, using 10 cc. samples to which an excess of KI and acetic acid had been added. For the determination of free NaOH or HOCl , chlorides, and CO_2 in the chlorinated solutions, 25 cc. samples were treated with 10 cc. of H_2O_2 to change NaOCl and HOCl to NaCl and HCl . When the evolution of gas was complete, 10 cc. portions were titrated for acid or alkali, and 5 cc. portions were titrated for chlorides by Volhard's method. CO_2 was estimated by the method of Van Slyke. Corrections were made for the acidity of the H_2O_2 added and for the dilution by the H_2O_2 . The figures charted are thus corrected.

Experiments with Sodium Hydroxide.

The titration figures per 10 cc. and the calculated composition of the solutions of the seven experiments of this group are given in Table III.

TABLE III.
Sodium Hydroxide Solutions.

Experiment No	Titration figures.				Calculated composition of solutions per 100 liters.				Sodium per 100 liters.	$\frac{\text{HOCl}}{\text{NaOCl}}$	
	Titration of 10 cc.		0.1 N thiosul- fate per 10 cc.	0.1 N NaCl per 10 cc.	NaOH		NaOCl				HOCl
	0.1 N HCl	0.1 N NaOH			gm. mols.	gm. mols.	gm. mols.	gm. mols.			
	cc.	cc.	cc.	cc.	gm. atoms						
I. (a) Original solution.....	27.40*	—	0.00	0.00	27.4				27.4		
(b) End-point to powdered phenolphthalein.....	0.45*	—	27.00	13.65	0.4	13.5	13.6		27.5		
(c) “ “ alcoholic	—	9.95*	35.35	37.25		8.2	19.6	9.8	26.8		
II. (a) Original solution.....	26.85*	—	0.00		26.9				26.9		
(b) End-point to powdered phenolphthalein.....	0.40*	—	26.65		0.4	13.3	13.3		27.0		
(c) “ “ alcoholic	—	11.80*	38.20			7.3	19.6	11.8	26.9		
III. (a) Original solution.....	27.80*	—	0.00	0.00	27.8				27.8		
(b) End-point to alcoholic phenolphthalein.....	—	5.75*	32.75	33.40		10.8	17.0	5.7	27.8		
IV. (a) Original solution.....	27.70†	—	0.00	0.00	27.7				27.7		
(b) End-point to powdered phenolphthalein.....	0.25†	—	27.26	27.80	0.2	13.6	14.2		28.0		
(c) “ “ <i>o</i> -cresolphthalein.....	—	2.54†	30.09	30.90		12.7	15.9	2.5	28.6	0.197	
V. (a) Original solution.....	27.70†	—	0.00	0.00	27.7				27.7		
(b) End-point to powdered phenolphthalein.....	0.39†	—	27.35	27.70	0.4	13.7	14.0		28.1		
(c) “ “ <i>o</i> -cresolphthalein.....	—	1.47†	29.40	29.92		13.2	15.2	1.5	28.4	0.114	

VI. (a) Original solution.....	12.80†	—	0.00	0.00	12.8	6.01	7.04	0.45	12.8	0.075
(b) End-point to <i>o</i> -cresolphthalein.....	—	0.45†	12.92	13.50					13.1	
VII. (a) Original solution.....	12.80†	—	0.00	0.00	12.8	6.07	7.14	0.49	12.8	0.081
(b) End-point to <i>o</i> -cresolphthalein.....	—	0.49†	13.12	13.70					13.2	

* Indicator used, phenolphthalein.

† " " sodium alizarin sulfonate.

TABLE IV.
Sodium Carbonate Solutions.

Experiment No.	Titration figures.					$\frac{\text{HOCl}}{\text{NaOCl}}$ ratios used in calculation.	Calculated composition of solutions per 100 liters.					Sodium per 100 liters.	Calculated hydrogen ion concentration.	pH	
	0.1 N HCl per 10 cc. to		0.1 N thiosulfate per 10 cc.	0.1 N NaCl per 10 cc.	CO ₂ per 10 cc.		NaOH	Na ₂ CO ₃	NaHCO ₃	NaOCl	NaCl				HOCl
	Phenol- phthalein.	Methyl orange.													
VIII. (a) Original solution.....	27.00	55.00	0.00	0.00	67.00		27.00	1.00					55.5		
(b) End-point to powdered phenol- phthalein.....	14.30	39.70	14.90	15.20	62.00		14.30	11.10	7.40	7.8			54.9	10.2	
IX. (a) Original solution.....	27.35	53.85	0.00	0.00	64.00		0.40	26.90					54.2		
(b) End-point to powdered phenol- phthalein.....	14.70	41.90	12.50	12.96			14.70	12.50	6.20	6.70			54.3	10.2	
(c) End-point to o-cresolphthalein.....	4.20	27.00	27.00	27.30	42.00		6.40	16.40	11.30	13.80	2.20		54.3	9.7	
							5.15	17.65	12.55	13.80	0.95		54.3	9.6	
X. (a) Original solution.....	27.10	55.80	0.00	0.00			27.10	1.60					55.8		
(b) End-point to powdered phenol- phthalein.....	13.20	41.30	13.78	14.50			13.20	14.90	6.89	7.61			55.8	10.1	
(c) End-point to o-cresolphthalein.....	3.89	27.00	27.73	28.80			6.16	16.95	11.60	14.93	2.27		55.8	9.7	
							4.86	18.25	12.90	14.93	0.97		55.8	9.55	

The method of calculation will be given in detail for Experiment I. For convenience all concentrations are given as gram molecules per 100 liters.

Experiment I.

Original NaOH Solution.—From the titration of the alkalinity of the original solution the initial concentration of 27.4 gm. molecules per 100 liters of NaOH was calculated.

NaOH Solution Chlorinated Till It No Longer Colors Powdered Phenolphthalein.—This solution required, after decomposition of the NaOCl by H_2O_2 , 0.45 cc. 0.1 N HCl per 10 cc., and this must be attributed only to free NaOH. The sodium hypochlorite was determined by titration with thiosulfate. The NaCl was taken as the difference between the total NaCl found and the NaOCl which had been converted by the H_2O_2 into NaCl and was therefore included in the Volhard estimation.

NaOH Solution Chlorinated to End-Point to Alcoholic Phenolphthalein.—This solution upon titration was acid and required 9.95 cc. of 0.1 N NaOH. The titratable acid, which it will be remembered was measured after decomposition of the NaOCl to NaCl and of the HOCl to HCl by H_2O_2 , must have been due to HCl derived from HOCl and to such oxidized acids, HClO_2 , HClO_3 , and HClO_4 , as may have been formed. In Experiment Ic (see Table III) the chlorides found are in excess of what would be produced in accordance with the equations given above, which call for as many molecules of chlorides as of NaOCl and HOCl combined. However, chloride figures are always somewhat higher than the total hypochlorite. Foerster and Dolch have shown that there is some liberation of oxygen from NaOCl with the formation of NaCl. This is, in part, the explanation of the excess of chlorides. In addition, at the less alkaline end-points of *o*-cresolphthalein and alcoholic phenolphthalein the formation of chloric and other oxidized acids, which is associated with the formation of NaCl, accounts for a part of this excess of chlorides. It is not possible to calculate from the measurements available in our experiments the precise amounts of these oxidized acids present, but the small excess of chloride over that calculated indicates that the quantities must be small, probably less than 0.3 gm. molecules

per 100 liters, even in those solutions which have the largest amount of HOCl. Such small quantities can be disregarded and we therefore calculate the entire acid found as HOCl. This subtracted from total hypochlorite gives the NaOCl, and the total hypochlorite subtracted from the total chloride gives the NaCl content of the solution before the addition of peroxide. As a check upon the analyses, the sodium content in gm. atoms per 100 liters has been calculated as the sum of the Na in the NaCl and NaOCl, and is given in a separate column. These figures should be theoretically constant for each experiment. From these experiments it will be seen that at the end-point of powdered phenolphthalein all of the hypochlorite is present as NaOCl and a trace of free alkali still persists. At the end-point to *o*-cresolphthalein a small fraction of hypochlorite is present as the acid so that the ratio $\frac{\text{HOCl}}{\text{NaOCl}}$ varies from 0.075 to 0.197.

Experiments with Sodium Carbonate.

In Table IV are given the results of the experiments made with sodium carbonate solutions. The titration of the alkali was done by the phenolphthalein-methyl orange method in order that the carbonate and bicarbonate might be calculated separately. In the original solutions and at the end-point to powdered phenolphthalein there are no acids other than the NaHCO_3 . However, we know from Experiments IV to VII that in the chlorinated solutions at the end-point to *o*-cresolphthalein there is an appreciable amount of HOCl present which will have been converted by the peroxide into HCl before the phenolphthalein-methyl orange titration is carried out. Correction in the latter titration figures must therefore be made for this added HCl. This has been done on the assumption that the $\frac{\text{HOCl}}{\text{NaOCl}}$ partition in the carbonate solution, within the range of hypochlorite concentration used, is the same for a given indicator end-point as in the sodium hydroxide solutions. By taking, therefore, the extreme values obtained from this ratio in the hydroxide experiments and using each of these ratios in succession in calculating the carbonate experiments at the *o*-cresolphthalein end-point, the limits of composition of the latter solutions can be estimated. This calculation

may be illustrated from Experiment IX (Table IV). Total hypochlorite = 13.5. Using ratio $\frac{\text{HOCl}}{\text{NaOCl}} = 0.197$, then of the total hypochlorite $11.3 = \text{NaOCl}$ and $2.2 = \text{HOCl}$. Correcting the alkali titration figures for the HOCl we obtain with phenolphthalein $\frac{1}{2} \text{Na}_2\text{CO}_3 = 4.2 + 2.2 = 6.4$; with methyl orange $\text{NaHCO}_3 + \text{Na}_2\text{CO}_3 = 27 + 2.2 = 29.2$. From these we derive $6.4 \text{ Na}_2\text{CO}_3 + 16.4 \text{ NaHCO}_3$.

The pH values are calculated from the $\frac{\text{NaHCO}_3}{\text{Na}_2\text{CO}_3}$ ratios by McCoy's formula. It will be seen that the pH at this end-point to powdered phenolphthalein lies between 10.2 and 10.1. The end-point to *o*-cresolphthalein lies between 9.2 and 9.7. The solution with the most alkaline end-point to this indicator had twice the hypochlorite concentration of the solution with the least alkaline. Since the shifting of the end-point with these indicators from the neutral point toward the alkaline side is the result of the oxidizing action of the hypochlorite it would be expected that the displacement of the end-point will be greater in a stronger hypochlorite solution. At the end-point to alcoholic phenolphthalein the solution is so unstable as to make the analyses too uncertain for calculation in this manner. It is probable, however, by analogy from the other two end-points and the pH values calculated for them and by consideration of the relation between the thiosulfate titration figures for the three end-points as given in Table II, that the end-point of alcoholic phenolphthalein is about as much less alkaline than that of *o*-cresolphthalein as the latter is less alkaline than that of powdered phenolphthalein; that is, for alcoholic phenolphthalein the end-point lies near a pH of 8.5 to 8.8.

The marked loss which occurs in the CO_2 from the carbonate solution during the introduction of chlorine is shown by the analyses in Experiments VIII and IX.

DISCUSSION.

It is evident from the results obtained in these experiments that in these solutions a much greater degree of alkalinity is required to produce color with powdered phenolphthalein than is required to

give a flash of color with alcoholic phenolphthalein solution. The color production in these hypochlorite solutions is probably dependent upon the relative rates of bleaching, on the one hand, and of formation of red alkaline phenolphthalein on the other. The color intensity is due to the amount of dye present in solution and to the degree of alkalinity. In using powdered phenolphthalein in the less alkaline solutions the rate of oxidation is so rapid that the minute amount of dye that dissolves in the solution is bleached before color can be detected. As the solution becomes more alkaline the intensity of color increases and also apparently the rate of oxidation decreases until a point is reached where the rate of color production is greater than the rate of the bleaching. This is at a pH of about 10.2.

When one adds a small drop of alcoholic phenolphthalein solution to a hypochlorite solution the color is discharged so rapidly that the eye cannot see it. If several drops are added rapidly—as was always done in these experiments—the amount of phenolphthalein changed to the color product is sufficient to persist for an appreciable time. The flash of color with alcoholic phenolphthalein is not therefore an exact end-point, for it may easily be shifted through a considerable range. It is also clear that sodium hypochlorite solutions such as are used in clinical work (Dakin's solution) when prepared so as to just fail to color powdered phenolphthalein are still strongly alkaline in reaction. In fact, a truly neutral solution or even one sufficiently alkaline to color alcoholic phenolphthalein would contain so large a proportion of HOCl as to be quite unstable and unsuitable for clinical use. We have found that a solution intermediate in alkalinity between the end-points of alcoholic and solid phenolphthalein gives the end-point to *o*-cresolphthalein described in this paper. This solution is still sufficiently alkaline to be stable for practical purposes. It is possible that such a solution may be tolerated upon skin and other tissue surfaces which would not bear the more alkaline solution at the end-point to powdered phenolphthalein. Unquestionably in clinical use hypochlorite solutions of widely varying reaction have in the past been used in spite of the fact that all failed to give a color with powdered phenolphthalein. It would seem desirable, at least in the more careful studies of the effects of these solutions, to define somewhat more closely the locations of their pH. This may be

done within certain limits by noting the behavior of the solution with *o*-cresolphthalein as well as with powdered phenolphthalein; in the less alkaline ranges even alcoholic phenolphthalein may be used for this purpose.

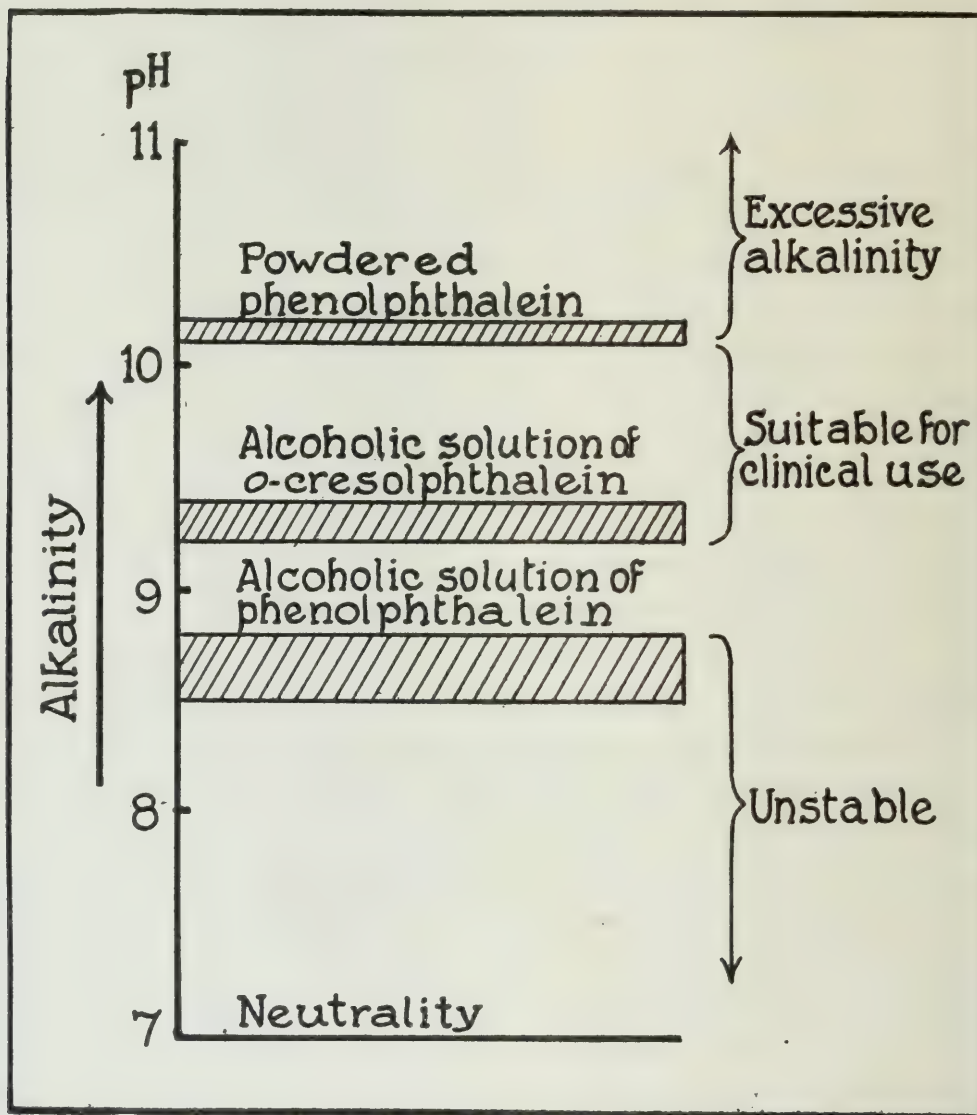


FIG. 1. Approximate hydrogen ion concentrations at end-points to powdered phenolphthalein, alcoholic solution of phenolphthalein, and alcoholic solution of *o*-cresolphthalein in 0.5 per cent sodium hypochlorite solutions.

The relation between the hydrogen ion concentration at these indicator end-points can be readily visualized by means of the accompanying chart (Fig. 1).

Application to the Preparation of Dakin's Solution.

For a consideration of the carbonate concentration employed in the above experiments it is evident that a hypochlorite solution having a pH of about 9.4 can be readily prepared by passing chlorine gas through a sodium carbonate solution of an initial concentration of 14 gm. to the liter (13.2 molecules per 100 liters) till sodium hypochlorite is formed in a concentration of 0.5 per cent as shown by titration with thiosulfate. A solution so prepared has proved most satisfactory for clinical purposes in this hospital.

SUMMARY.

1. It is shown that the end-points of indicators in dilute sodium hypochlorite solutions are different from the end-points of the same indicators in ordinary solutions.

2. A method is described for studying the hydrogen ion concentrations of dilute sodium hypochlorite solutions by means of the end-points of powdered phenolphthalein, of phenolphthalein in alcoholic solution, and of *o*-cresolphthalein in alcoholic solution.

3. The end-point to powdered phenolphthalein in a 0.5 per cent sodium hypochlorite solution is at a pH of about 10.1. All of the hypochlorite is present as the salt (NaOCl).

4. The end-point to alcoholic solution of *o*-cresolphthalein in 0.5 per cent sodium hypochlorite solution is at a pH of about 9.3. The same end-point in a 1 per cent sodium hypochlorite solution is at a pH of about 9.6. Of the total hypochlorite in these solutions from 7 to 17 per cent is present as HOCl. This less alkaline solution is sufficiently stable to be satisfactory for clinical use.

5. The end-point to alcoholic solution of phenolphthalein in similar solutions cannot be precisely estimated by our method owing to the rapid decomposition of the HOCl, which constitutes from 35 to 60 per cent of the total hypochlorite of such solutions. The pH is probably, however, about 8.5 to 8.8. This solution is highly unstable because of the low alkalinity, and is also for other reasons unsuitable for clinical use.

6. It is suggested that for certain purposes the hydrogen ion concentration of dilute hypochlorite solutions be defined more closely

than has been the custom in the past. For this purpose the end-points of the indicators established above are of value.

7. For preparing sodium hypochlorite solution (Dakin's solution) from chlorine and sodium carbonate, a method is outlined which secures the required hypochlorite concentration and also the desired alkalinity, simply by using definite amounts of carbonate and chlorine.

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RESULTS OF PROPHYLACTIC INOCULATION AGAINST PNEUMOCOCCUS IN 12,519 MEN.

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The success of prophylactic vaccination against typhoid fever as demonstrated by the results obtained in the United States Army naturally suggests the effort to combat other prevalent and serious diseases by the same method. Among such diseases pneumonia easily ranks first. During the past winter it has been responsible for probably 80 per cent of the deaths in the various training camps in this country, and though the streptococcus has been the causative agent in many of these cases the pneumococcus has also played a prominent part.

Animal experiments have shown that it is easy to produce active immunity to pneumococci by the injection of small doses of dead organisms even in animals as susceptible as the mouse and rabbit, and this immunity persists for a considerable time. It is theoretically possible, therefore, to immunize man to the fixed types of pneumococci by injection of dead cultures.

The only place to our knowledge where preventive inoculation against pneumonia has been hitherto attempted has been among the workers in the mines in South Africa, where the disease occurs with great frequency and causes the death of large numbers of native workmen. The first inoculations on a large scale were carried out by Sir Almroth Wright (1) in 1911 and 1912. However, in these experiments no attention was paid to differences in types of pneumococci and this fact renders the interpretation of the results of the inoculations extremely difficult. In 1913 Dochez and Gillespie (2) published a classification of pneumococci and Lister (3) independently reported shortly afterwards a similar classification

of the pneumococci encountered in South Africa. Lister then undertook an experimental study of prophylactic inoculation against the various types of pneumococci in animals and man. He demonstrated that immunity can be produced in man against at least certain ones of these types either by subcutaneous or intravenous injection, more readily by the latter. He found that subcutaneous inoculation of 40 billion cocci of the strains he employed caused little if any toxic reaction in the guinea pig, rabbit, or man, and intravenous inoculation of 20 billion in the rabbit and 40 billion in man gave rise to but slight toxic reaction. On the basis of these experiments Lister undertook the prophylactic inoculation of large groups of miners against pneumococcus and has recently reported the results of his experiments. He at first advocated inoculation at 7 day intervals, each dose to consist of 6 billion cocci of each type against which immunity was desired. Subsequently he greatly reduced this dosage and gave three subcutaneous inoculations at 7 day intervals, each injection consisting of 2 billion of each type.

The workers in three different mines, the Crown, Premier Diamond, and De Beers Diamond, were inoculated with a vaccine composed of the three types of pneumococcus which were most prevalent in these mines. They were known as Types A, B, and C. Types B and C correspond to Types II and I, respectively, in Dochez and Gillespie's classification. Type A has not been encountered in America. In the De Beers Diamond Mine a fourth group was added, called Type H. In the De Beers experiment 1 billion of Type H was added to each injection, making a total dosage at each injection of 7 billion. The vaccinated miners were then observed over a period of 6 to 12 months and in all three mines a definite decrease in the incidence and mortality rate from pneumonia was observed. In the case of the Crown Mines, every case of pneumonia which occurred among the vaccinated individuals was studied bacteriologically and the type of pneumococcus determined. No cases of the types against which the men had been vaccinated (Types A, B, and C) developed during the 9 months of observation. Lister contends that this fact, namely the alteration of a relative group prevalence by means of specific group inoculation, is a more critical test of the efficacy of pneumonia prophylaxis than the simultaneous comparison of pneumonia rates in inoculated and uninoculated (control) groups when the comparison is based upon the erroneous assumption that all cases of disease due to the pneumococcus are bacteriologically indistinguishable. He emphasizes the probability that the protection of a considerable part of the community by inoculation lessens the number of carriers, and perhaps the virulence of the strains found in the community, and hence confers a definite benefit upon the uninoculated group which would affect the use of this group as controls in a statistical sense. Lister reported no unpleasant effects from the vaccine.

Bacteriological study of the first hundred cases of pneumonia occurring at Camp Upton showed that about 70 per cent were due to the pneumococcus and of these, about 50 per cent were caused by Type

I, II, or III. In consideration of this fact and of the results achieved by Lister in South Africa, it seemed desirable to employ prophylactic vaccination against the pneumococcus at Camp Upton. At the outset it was decided to incorporate only Types I, II, and III in the vaccine. Vaccination against Type IV was obviously not feasible on account of its many varieties. The preliminary problems to be determined were: First, how large a dose of such a vaccine could be administered without producing a severe reaction? Second, would the dose, injected three or four times subcutaneously, produce an efficient and demonstrable immunity?

Preliminary Experiments.

For the preparation of the vaccine preliminary experiments showed that agar media were unsatisfactory because of the small yield of organisms. Experiments in immunizing rabbits with pneumococci grown on a variety of media indicated that those obtained from 0.5 per cent glucose broth were as effective antigenically as those obtained from agar media or from plain broth, and as the yield from glucose broth is much more abundant this medium was adopted. To prevent autolysis, incubation was stopped after 12 to 14 hours. Centrifuging was carried out by the use of a continuous feed type of centrifuge (Sharpless laboratory centrifuge) which will take 8 to 12 liters of broth per hour and give a clearer supernatant fluid than is usually obtained from the bucket type of centrifuge. The cultures were killed before centrifuging by heating to 53°C. for $\frac{1}{2}$ hour. This conserves the antigenic effects of the culture. After centrifuging, the organisms were suspended in normal saline solution, shaken to secure even distribution, standardized by dilution and comparison of the opacity with known suspensions of pneumococci, and again heated at 55°C. for $\frac{1}{2}$ hour. Sterility of the vaccine was established by aerobic and anaerobic cultures and by subcutaneous inoculation into guinea pigs and intraperitoneal inoculation into mice. Tricresol was added to a concentration of 0.3 per cent as a preservative.

In order to determine the optimum dosage and interval of injection inoculations were given to forty-two adult volunteers in varying dosage and at different intervals, and the effect was gauged by means of tests made upon the serum taken 8 days after the last in-

jection. The character of the local and general reactions to the inoculation was also observed. The tests applied to the serum were the agglutination titer against the three types included in the vaccine and the protective power of the sera against these types injected into mice.

The agglutination titer was carried out with 24 hour plain broth cultures of the same strains used in preparing the vaccine. The serum dilutions used were 1:1, 1:3, 1:10, and occasionally 1:30. The tubes were incubated for 2 hours at 37°C. in a water bath, given a first reading, placed in the ice box over night, and then given the final reading. The results charted are based on the final reading.

For the protection experiments the method described by Dochez (4) was employed. A plain broth passage culture from the heart's blood of a mouse was used for each of the three strains employed in preparing the vaccine. Several dilutions of these three cultures were made with sterile broth so that 0.5 cc. of the dilutions would contain respectively 0.01, 0.001, 0.0001, 0.00001, and 0.000001 cc. of original culture. 2 parts of each serum were diluted with 3 parts of normal salt solution so that 0.5 cc. of the diluted serum contained 0.2 cc. of serum. With each serum mice were injected intraperitoneally with some or all of the following mixtures of diluted serum and diluted cultures.

- Mouse 1, 0.5 cc. of diluted culture (equals 0.01 cc. of culture of Type I) + 0.5 cc. of diluted serum.
- Mouse 2, 0.5 cc. of diluted culture (equals 0.001 cc. of culture of Type I) + 0.5 cc. of diluted serum.
- Mouse 3, 0.5 cc. of diluted culture (equals 0.0001 cc. of culture of Type I) + 0.5 cc. of diluted serum.
- Mouse 4, 0.5 cc. of diluted culture (equals 0.01 cc. of culture of Type II) + 0.5 cc. of diluted serum.
- Mouse 5, 0.5 cc. of diluted culture (equals 0.001 cc. of culture of Type II) + 0.5 cc. of diluted serum.
- Mouse 6, 0.5 cc. of diluted culture (equals 0.0001 cc. of culture of Type II) + 0.5 cc. of diluted serum.
- Mouse 7, 0.5 cc. of diluted culture (equals 0.01 cc. of culture of Type III) + 0.5 cc. of diluted serum.
- Mouse 8, 0.5 cc. of diluted culture (equals 0.001 cc. of culture of Type III) + 0.5 cc. of diluted serum.
- Mouse 9, 0.5 cc. of diluted culture (equals 0.0001 cc. of culture of Type III) + 0.5 cc. of diluted serum.

As controls six mice were injected intraperitoneally as follows:

- Mouse 1, 0.5 cc. of diluted culture (equals 0.00001 cc. of culture of Type I) + 0.5 cc. of normal saline solution.
 Mouse 2, 0.5 cc. of diluted culture (equals 0.000001 cc. of culture of Type I) + 0.5 cc. of normal saline solution.
 Mouse 3, 0.5 cc. of diluted culture (equals 0.00001 cc. of culture of Type II) + 0.5 cc. of normal saline solution.
 Mouse 4, 0.5 cc. of diluted culture (equals 0.000001 cc. of culture of Type II) + 0.5 cc. of normal saline solution.
 Mouse 5, 0.5 cc. of diluted culture (equals 0.00001 cc. of culture of Type III) + 0.5 cc. of normal saline solution.
 Mouse 6, 0.5 cc. of diluted culture (equals 0.000001 cc. of culture of Type III) + 0.5 cc. of normal saline solution.

The time of injection of each mouse was noted and the number of hours to time of death recorded. When a mouse had survived over 140 hours it was recorded as "survived." Upon dying the mice were autopsied and films made from the peritoneal exudate and stained to determine the presence of Gram-positive diplococci. From the control mice, upon dying, cultures were made from the heart's blood and the type was verified by agglutination. The range of survival exhibited in the controls throughout the tests is shown in Table I.

TABLE I.
Mouse Controls for Protection Tests.

	Type I.		Type II.		Type III.	
	0.00001 cc.	0.000001 cc.	0.00001 cc.	0.000001 cc.	0.00001 cc.	0.000001 cc.
Minimum.....	28*	31	18	16	14	33
Maximum.....	36	36	40	32	36	45

*The figures indicate the hours of survival of each mouse after injection.

All inoculations except in Individual 9 were given subcutaneously. The first one inoculated, Individual 9, received two subcutaneous inoculations of 16 billion cocci of each type at each inoculation, 4 days apart, followed 4 days later by one-tenth this dose intravenously. An extensive area of redness with some tenderness followed the subcutaneous inoculations, reaching the maximum in 48 hours but without incapacitating the subject; there was no accompanying general

reaction. The intravenous injection was followed 48 hours later by a slight malaise and a temperature of 100.4°F. of a few hours' duration.

All other inoculations were given subcutaneously as this was considered the only method feasible for use later on a large scale in the camp.

Individuals 1 to 8 (Table II) received a first dose subcutaneously of 8 billion cocci of each type. In four adults a moderate local reaction resulted. In two, however, the local reaction was severe, leading

TABLE II.

Individuals Receiving a Single Large Inoculation.

Individual No.	Total dosage in billions.			Reaction.		Tests on sera 7 days after injection.																	
						Agglutination.						Mouse protection.											
	Type I.					Type II.			Type III.	Type I.			Type II.			Type III.							
	Local.	General.	1:1	1:10	1:30	1:1	1:10	1:30	1:1	1:10	0.01 cc.	0.001 cc.	0.0001 cc.	0.01 cc.	0.001 cc.	0.0001 cc.	0.01 cc.	0.001 cc.	0.0001 cc.				
1	8	8	8	Sl.*	N.	++	-	-	++	-	-	-	-	-	S.	S.	S.	27	S.	S.	25	28	52
2	8	8	8	Mod.	"	+	-	-	++	-	-	-	-	-	30	103	"	21	"	"	25	37	S.
3	8	8	8	"	"	-	-	-	++	-	-	-	-	-	20	73	"	21	"	"	25	26	57
4	8	8	8	Sev.	Sl.	-	-	-	++	-	-	-	-	-	18	29	50	14	"	"	17	24	31
5	8	8	8	Mod.	Sev.	-	-	-	-	-	-	-	-	-	30	S.	S.	21	80	"	19	27	S.
6	8	8	8	Sev.	"	-	-	-	-	-	-	-	-	-	17	"	41	10	54	"	12	27	51
7	8	8	8	Mod.	Sl.	-	-	-	+	-	-	-	-	-	17	29	50	12	80	"	10	51	S.
8	8	8	8	Sl.	Sev.	-	-	-	-	-	-	-	-	-	20	39	S.	22	44	"	24	29	43

* In the tables N. indicates none, Sl., slight, Mod., moderate, Sev., severe, and Inf., infiltration. The figures indicate the hours of survival of each mouse after injection. S. indicates survival over 140 hours.

to a reddening and swelling of the area from the shoulder almost to the wrist, persisting 3 days and rendering use of the arm during this time difficult. In three adults a chill with some nausea, marked malaise, and slight rise of temperature occurred in the first 24 hours. It was concluded that reactions of this type were too severe to be permissible in a large scale vaccination. A series of persons (Nos. 10 to 21, 25, and 27, Table III) was accordingly inoculated with four or five injections of much smaller doses at 3 to 7 day intervals (except

in two cases in which the interval between the first two doses was 2 days). Inasmuch as the suggestion had been made that Type III was especially prone to induce severe local reactions this type was omitted from one of the inoculations in Individuals 10 to 16 and 27. No difference could be observed between the local reaction in these cases when Type III was omitted. The dosage used in Individuals 12 to 16 was as follows:

Day.	No. of billions of Type I.	No. of billions of Type II.	No. of billions of Type III
1	1	1	1
4	2	2	2
7	4	4	4
13	6	6	

The dosage of each type in other individuals was as follows: Individual 17: first injection 1 billion; second injection 2 billion; third injection 3 billion; fourth injection 4 billion. Individuals 18 and 20: first injection 1 billion; second injection 2 billion; third injection 2 billion; fourth injection 3 billion. Individuals 19 and 21: first injection 1 billion; second, third, and fourth injections each 2 billion. Individual 25 received only half as much of Type III as of Types I and II at each injection. Of these fourteen adults, marked local reactions occurred after at least one of the inoculations in four instances but not severe enough to incapacitate the individual. In none of these instances was there any severe general reaction. These doses appeared to be such as could be satisfactorily employed on a large scale without unduly incommoding a command. Still smaller doses were employed in Individuals 32, 33, 36, 37, and 38. These were without any severe local reactions and with moderate or no general reaction.

The experimental work of Cole and Moore (5) showed that in rabbits an immunity could be more rapidly induced by small daily intravenous inoculations of antigen than by much larger intravenous inoculations at longer intervals. To test the applicability of this principle to subcutaneous injections in man, four adults (Nos. 39 to 42) were given daily subcutaneous injections of 1 billion cocci of each type for 7 days. These injections were associated with only the mildest local reactions and with no constitutional reaction. The

TABLE III.

Cases Receiving Multiple Doses of Varying Amounts.

Individual No.		No. of injections.	Reaction.		Agglutination.												Mouse protection.												
					8 days after last injection.												Before vaccination.						8 days after last injection.						
Total dosage in billions.			Reaction.		Before vaccination.				Type I.				Type II.				Type III.				Type I.			Type II.			Type III.		
					Type I.	Type II.	Type III.	Type I.	Type II.	Type III.	Type I.	Type II.	Type III.	Type I.	Type II.	Type III.	Type I.	Type II.	Type III.	Type I.	Type II.	Type III.							
			Local.	General.	1:1	1:3	1:10	1:30	1:1	1:3	1:10	1:30	1:1	1:3	1:10	1:30	1:1	1:3	1:10	1:30	0.001 cc.	0.001 cc.	0.001 cc.	0.01 cc.	0.001 cc.	0.001 cc.	0.001 cc.		
9	3	4	34	34	Mod.	Sl.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
10	5	4-2	12	12	6	Sev.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
11	5	4-2	12	12	6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
12	4	6-3	13	13	7	Sl.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
13	4	5-3	13	13	7	Mod.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
14	4	4-3	13	13	7	Sl.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
15	4	5-3	13	13	7	Mod.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
16	4	6-3	13	13	7	Sl.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
17	4	5-3	10	10	10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
18	4	4-3	8	8	8	Mod.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
19	4	4-3	7	7	7	Sev.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
20	4	4-3	8	8	8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
21	4	4-3	7	7	7	Mod.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		

22	3	7	6	6	5	Inf.
23	3	7	6	6	5	"
24	3	7	6	6	5	"
25	3	7-4	7	7	3½	N.
26	3	7	6	6	5	Inf.
27	3	3	9	9	3	Sl. to Sev.
28	3	7	6	6	5	Inf.
29	3	7	6	6	5	"
30	3	7	6	6	5	"
31	3	7	6	6	5	"
32	4	7-4	4	4	4	Mod. N.
33	4	7-4	4	4	4	"
34	2	7	3	3	3	Inf.
35	2	7	3	3	3	"
36	3	7	2½	2½	2½	Mod. N.
37	3	7	2½	2½	2½	" Mod.
38	3	7	2½	2½	2½	Sl. "
39	7	1	7	7	7	" N.
40	7	1	7	7	7	" "
41	7	1	7	7	7	" "
42	7	1	7	7	7	" "

immune body response in the sera of these various groups is shown in Tables II and III.

From sixteen persons serum was obtained before commencing the vaccination. In none of these was there any agglutinin demonstrable against any of the three types and in only one instance (No. 9) were there any survivals in the mouse protection test, in this case against Type I. On the other hand, 8 days after the vaccination agglutinins to some extent were demonstrable in twenty-seven of the forty-two individuals studied and a definite degree of protective power as shown by the survival of at least some of the mice in all of the forty cases in which the mouse protection test was performed.

The degree of agglutinin production and of mouse protection varies so greatly in the individual cases in any one group that it becomes difficult to draw positive deductions as to the merits of the various methods of administration employed. This difficulty is indicated if we group the persons according to dosage and number of inoculations and tabulate the percentage of positive agglutinations at each titer and of mouse survivals for each dose of culture for these groups. This we have done in Table IV. From a study of this table the conclusion seems justified that the first group (Adults 10 to 21) which comprises those receiving the largest total dosage exhibits definitely the best response as judged by both agglutination and the mouse protection test. The second group (Adults 32, 33, 36, 37, and 38) received the smallest total dosage and gave definitely less satisfactory response. A comparison of the third group (Adults 1 to 8), the fourth group (Adults 18 to 21, 25, and 27), and the fifth group (Adults 39 to 42), all receiving practically the same total dosage but in the third group given in a single large dose, in the fourth in three or four moderate doses at 3 to 7 day intervals, and in the fifth in seven very small daily doses, shows no definite difference that can be detected in the response of the three groups. The conclusions that we draw from these experiments are that the immune response as measured by these tests will depend on the total dosage of vaccine and is little influenced by the number of doses into which this quantity is divided. The difference in toxic reaction, however, in these three groups was definite. The small daily doses gave hardly any reaction. The single very large dose gave rise to several severe local and constitutional reactions.

TABLE IV.

Summary by Percentages of Agglutination and Protection Tests in Sera.

Group.	Percentage of positive agglutinin reactions.						Percentage of mouse survivals in protection test.						
	Type I.			Type II.			Type I.			Type II.			Type III.
	1:1	1:3	1:10	1:1	1:3	1:10	0.01 cc.	0.001 cc.	0.0001 cc.	0.01 cc.	0.001 cc.	0.0001 cc.	0.0001 cc.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Group 1* (Individuals 10 to 21).....	67	75	33	58	58	17	12	54	82	14	60	82	18
“ 2 (“ 32, 33, and 36 to 38).....	40	20	0	20	20	0	0	25	75	0	25	75	25
Group 3 (Individuals 1 to 8).....	25		0	62		0	12	37	62	0	50	100	37
“ 4 (“ 18 to 21, 25, and 27).....	50	50	0	33	33	0	0	40	40	0	25	100	0
Group 5 (Individuals 39 to 42).....	0	25	25	25	25	0	0	50	50	0	67	75	0
“ 6 (“ 22 “ 24, 26, 28 to 31, 34, and 35).....	67	44	0	44	22	11	11	40	90	10	50	70	20

* Group 1 received a total dosage of from 7 to 13 billion cocci of each type. Group 2 received a total dosage of from 2½ to 4 billion cocci of each type. Groups 3, 4, and 5 received a total dosage of from 7 to 9 billion cocci of each type, Group 3 in a single large injection, Group 4 in three or four moderate injections at 3 to 7 day intervals, and Group 5 in seven small daily injections. Group 6 received a total dosage of 3 to 6 billion cocci of each type and developed infiltrations.

Vaccinations of Troops.

On the basis of these preliminary experiments we adopted for the larger scale vaccinations a moderate total dosage of 6 to 9 billion cocci of each type and administered this in four small doses at weekly intervals. By this method we expected to secure a definite immune response in the vaccinated individuals with a minimum of severe reactions and without undertaking a larger number of injections than would be within the capacity of the medical staff of the camp to carry out.

The detailed dosage decided upon was:

1st inoculation:	Pneumococcus Type I	1	billion
	" " II	1	"
	" " III	1	"
		<hr/>	
	Total.....	3	"

2nd inoculation:	Pneumococcus Type I	2	billion
	" " II	2	"
	" " III	2	"
		<hr/>	
	Total.....	6	"

3rd inoculation:	Pneumococcus Type I	3	billion
	" " II	3	"
	" " III	1½	"
		<hr/>	
	Total.....	7½	"

4th inoculation: Same as 3rd inoculation.

The vaccine was made up in three different concentrations so that the dose in every case was 0.5 cc.

Altogether, 12,519 men were vaccinated, about 40 per cent of the mean strength of the command. A great majority of these received three or four inoculations. Some, however, had only one or two. The vaccine was given at 5 to 7 day intervals, except in one organization where unavoidable circumstances necessitated a lapse of 20 days between the first and second inoculations. In most cases the vaccine was administered in the arm.

Reactions.

The constitutional reactions to the pneumococcus inoculations were usually negligible. Out of the entire number vaccinated, only twenty-five men were sufficiently ill to remain in quarters or the hospital for a short time. This number would probably have been larger if the vaccination had been compulsory. As it was, those who were upset by the first or second inoculation were usually not given the third or fourth. The impression prevailed among the regimental surgeons that the reactions to pneumococcus vaccination were milder than those to typhoid vaccination. The troops, however, were in better physical condition when they received the pneumococcus vac-

cine than when the typhoid inoculations were given. In those who reacted severely the symptoms simulated an attack of influenza. The patient complained of general malaise, chilly sensations, fever, and muscular pains. In addition, a certain number of those who reacted severely had symptoms referable to the upper respiratory tract, such as coryza, sore throat, cough, and pain in the chest. A number of those who were suffering from infections of the air passages at the time of inoculation, claimed that their symptoms were

TABLE V.

Incidence of Infiltrations and General Reactions among the Vaccinated Troops.

Organization.	No. vaccinated (approximate).	Reactions.	
		Infiltrations.	General reaction (quarters or hospital).
305th Infantry.....	2,500	43	12
306th "	2,800	62	6
307th "	1,500	6	0
308th "	2,150	28	5
302nd Sanitary Train.....	700	3	1
367th Infantry (colored).....	1,500	1	0
304th Machine Gun Battalion.....	400	2	0
305th " " "	400	4	1
306th " " "	500	3	0
302nd Engineers.....	35	0	0
Base Hospital.....	25	0	0
Total.....	12,510	152 (1 in 82)	25 (1 in 500)

more marked after receiving the injection. Constitutional reaction to pneumococcus vaccination apparently develop more slowly than with typhoid inoculation, sometimes not appearing until 24 hours after the injection.

The local reaction to pneumococcus vaccination differs little, as a rule, from that to typhoid vaccination. At the point of inoculation an area of tenderness and induration develops, usually about 5 to 10 cm. in diameter. The area of induration is nearly always oblong and extends down the arm below the point of inoculation. The axillary glands are sometimes swollen and tender. The tenderness

and swelling at the point of inoculation rapidly decrease, however, and at the end of 3 or 4 days have usually disappeared.

An unexpected and somewhat troublesome complication arose in connection with the vaccination which at first gave us some concern; this was the development of a certain number of small infiltrations at the site of inoculation. They developed slowly, rarely coming to the stage of fluctuation before the 6th or 7th day after inoculation. In size they were usually 2 or 3 cm. in diameter, and in only one instance extended down deeper than the subcutaneous tissue. At first they were looked upon as the result of careless technique, but repeated cultures showed them to be invariably sterile. Some of them developed after the first inoculation, but a greater number followed the larger doses. In a few cases an infiltration developed with each inoculation given. This would have probably happened more frequently had the vaccination not been discontinued in those who developed the condition after the first or second dose. At first the regimental surgeons made an incision, but later it was found that the infiltrations would progress favorably if left alone. Altogether, 152 men developed the lesion (1 in every 82, Table V). They were fairly evenly distributed throughout the various organizations vaccinated, with the exception of the 1,500 vaccinated negroes, among whom only one developed. The infiltrations were tender and painful in the early stage of their development but later became cold and painless. They apparently developed from a hypersensitiveness to the pneumococcus or pneumotoxin. In the hope of discovering whether these individuals exhibited a more or less marked immune response in their sera, the sera of ten such persons were studied (Table III, Adults 22 to 24, 26, 28 to 31, 34, and 35). The percentile results are tabulated in Table IV as Group 6. Apparently the immune response in this group is analogous to that of the other individuals receiving the same total dose of vaccine. We would conclude, therefore, that there is no demonstrable difference in the degree of immune response by the tests we have used in individuals showing pronounced local reaction even to the extent of local infiltration described. Few of the men responding in this way had ever had pneumonia. With only one exception they did not occur among the colored troops who are especially susceptible to pneumonia. They

are not related in any way to the method of administration of the vaccine.

In order to test the relation of these severe reactions to the susceptibility to pneumotoxin, the following experiments were performed. Pneumotoxin was prepared by Cole's (6) method, which consists of growing pneumococci in plain broth; centrifuging and washing in normal saline solution; solution of the pneumococci in weak sodium taurocholate solution at 37°C.; dilution to one-tenth the bulk of the original broth culture. This pneumotoxin was diluted 1:10 with normal saline solution and injected intradermally to the amount of 0.1 cc. in a series of healthy volunteers. Little resulting reaction was noted in most individuals. However, in those who had shown severe local reactions to the vaccination or had developed infiltrations, an extensive areola developed about the pneumotoxin injection, reaching the maximum after about 24 to 36 hours and often being associated with considerable tenderness. The reaction to the pneumotoxin occurred regardless of the type used in its preparation, exhibiting no specificity for type. These findings suggest that the severe local reactions to the vaccine are due to an unusual sensitiveness to a pneumotoxin which is common to all the types of pneumococci.

Table V shows the number of men vaccinated in each organization and the incidence of infiltrations and severe general reactions in each.

Bacteriological Examination of Sputa.

Particular attention was directed to the bacteriological examination of the sputum in the cases of pneumonia that developed during the period of observation. In seven instances this examination, through some unavoidable circumstance, was not completed. In all the others, however, the sputum was examined and the predominant organism determined.

The Avery blood broth method (7) was used in a great majority of cases and in addition, whenever it was possible, a mouse was inoculated, or a direct culture from sputum was made on a blood agar plate. The following figures indicate the number of times each method was used:

1. Both mouse and blood broth.....	47
2. " blood broth and direct culture.....	23
3. Blood broth alone.....	108
4. Mouse alone.....	5

Results of Vaccination.

The vaccination of the troops began on February 4, 1918. The Division was transferred from Camp Upton about April 15, 1918. The following figures are based on the period extending from February 4 to April 15, about 10 weeks. The number of troops vaccinated was 12,519. The number of unvaccinated was approximately 19,481. The latter figure varied, of course, from day to day as new men came and others departed. The vaccinated men were in stable organizations where the personnel underwent little change.

Before discussing the results of vaccination, it is of interest to note the incidence of pneumonia previous to the beginning of vaccination in the various organizations which later received the vaccine and to compare this with the incidence of pneumonia at the same period among the organizations which did not later receive the vaccine. Previous to February 4, the day on which the experiment began, there had been 91 cases of pneumonia among the troops at Camp Upton. Of these, 29 occurred among the organizations which were subsequently vaccinated, while 43 cases occurred among the units which were later to be used as a control; 19 occurred among casualties not included later in either group. It will be seen from this that the cases of pneumonia were quite evenly divided between the 40 per cent of the troops which were to be vaccinated and the 60 per cent which were to be used for controls. Of the 29 pneumonias occurring among the organizations which were subsequently vaccinated, 9 were due to pneumococci of Type I, II, or III and 11 to *Pneumococcus* Type IV. Of the 43 pneumonias occurring among the control group, 16 were due to pneumococci of Type I, II, or III and 17 to *Pneumococcus* Type IV.

Furthermore, about 30 per cent of all these pneumonias were streptococcus cases and these, too, were fairly equally divided between the two groups of organizations, 9 occurring among the men vaccinated later, and 10 among the control group.

Incidence of Pneumonia among the Vaccinated Troops from February 4 to April 15, 1918.—There has been but one case of pneumonia due to pneumococcus of Type I, II, or III among the vaccinated troops during this period. This case, due to Type I pneumococcus, developed 24 hours after the first inoculation and therefore before protection could have been developed. During this period sixteen cases of other types of pneumonia occurred among the vaccinated troops (Table VI). There were nine Type IV pneumococcus cases; three of these had only received one injection of vaccine. The remaining seven cases were streptococcus infections, six due to the hemolytic streptococcus, and one to *Streptococcus viridans*. None of the pneumococcus cases died. The Type I case received Type I serum and

TABLE VI.

Incidence of Pneumonia among the Vaccinated Troops, February 4 to April 15, 1918.

Average strength of command, Feb. 4 to Apr. 15, 1918.....	32,000
No. of troops vaccinated against pneumonia.....	12,519 (40 per cent)
“ “ unvaccinated men (average).....	19,481 (60 “ “)
Incidence of pneumonia among the vaccinated troops, Feb. 4 to Apr. 15:	
Pneumococcus Type I (developed 24 hrs. after 1st injection).....	1
“ “ IV (3 cases receiving only 1 injection).....	9
<i>Streptococcus hæmolyticus</i>	6
“ <i>viridans</i>	1
<hr/>	
Total No. of pneumonias among the vaccinated troops.....	17

made an uneventful recovery. Most of the Type IV cases ran a mild course, so mild in some cases that the diagnosis of pneumonia was made only by the aid of the x-ray. Two of the streptococcus cases died, both being of the hemolytic group.

Incidence of Pneumonia among the Unvaccinated Troops from February 4 to April 15, 1918.—The unvaccinated fraction of the camp has been divided into two groups (Table VII). First, the old troops whose physical condition and resistance to infection were presumably the same as that of the vaccinated men. They constituted, numerically, about 75 per cent of the controls. Second, the new troops who consisted of newly drafted men coming into camp between February 26 and April 15 and who by reason of their lack of training were prob-

ably more susceptible to infection than the seasoned troops. These comprised about 25 per cent of the controls. Among the old troops there were eighteen cases of pneumonia due to *Pneumococcus* Type I, II, or III, and seventeen cases due to Type IV, making a total of

TABLE VII.

Incidence of Pneumonia among the Unvaccinated Troops, February 4 to April 15, 1918.

Type of pneumonia.	No. of cases of pneumonia
Old troops (75 per cent of control).	
Pneumococcus Type I.....	8 } " II..... 5 } 18 } " III..... 5 } 35 " IV..... 17 } 17 }
<i>Streptococcus hemolyticus</i>	45 }
" <i>viridans</i>	23 } 68
<i>B. influenzae</i>	1
Type undetermined.....	6
Total	110
New drafted men, Feb. 26 to Apr. 15 (25 per cent of control).	
Pneumococcus Type I.....	2 } " II..... 4 } 8 } " III..... 2 } 24 " IV..... 16 } 16 }
<i>Streptococcus hemolyticus</i>	27 }
" <i>viridans</i>	11 } 38
Type undetermined.....	1
Total.....	63
Total No. of Type I, II, and III pneumonias among the unvaccinated troops.	26
" " " " I, II, III, and IV " " " " " "	59
" " " streptococcus pneumonias among the unvaccinated troops.....	106
" " " pneumonias among the unvaccinated troops.....	173

thirty-five cases of pneumococcus pneumonia. There were sixty-eight streptococcus cases, forty-five of the hemolytic type and twenty-three of the non-hemolyzing type. There was one *Bacillus influenzae* case and six cases in which the type was not determined.

Among the new men the proportion was about the same. There were eight cases due to *Pneumococcus* Type I, II, or III. There were sixteen cases of pneumonia due to *Pneumococcus* Type IV, making twenty-four cases of pneumococcus pneumonia. There were thirty-eight cases of streptococcus pneumonia, twenty-seven of the hemolytic type and eleven of the non-hemolyzing type. There was one case in which the organism was not determined.

TABLE VIII.
Mortality Rates.

Type of pneumonia.	Deaths.	
Among unvaccinated troops.		
Pneumococcus.		per cent
Types I, II, and III.....	7	27
Type IV.....	6	18
<i>Streptococcus hæmolyticus</i>	26	36
“ <i>viridans</i>	4	12
Type undetermined.....	5	
Total.....	48	28
Annual pneumonia death rate per 1,000 for unvaccinated troops.....	12.8	
Among vaccinated troops.		
Pneumococcus.....	0	
<i>Streptococcus hæmolyticus</i>	2	
“ <i>viridans</i>	0	
Total.....	2	11.7
Annual pneumonia death rate per 1,000 for vaccinated troops.....	0.83	

A summary of the control shows that there were twenty-six cases of pneumococcus pneumonia of Type I, II, or III. There were thirty-three *Pneumococcus* Type IV cases, making a total of fifty-nine pneumococcus pneumonias. There was a total of 106 streptococcus pneumonias, 72 of which were of the hemolytic type and 34 of the *viridans*, or non-hemolyzing type. Altogether there were 173 pneumonias among the unvaccinated troops.

There were seven deaths among the *Pneumococcus* Type I, II, and III cases, or 27 per cent (Table VIII). There were six deaths among the *Pneumococcus* Type IV cases, or 18 per cent. Of the streptococcus cases, hemolytic type, twenty-six died, or 36 per cent. There were only four deaths among the *viridans* cases, the mortality being 12 per cent.

DISCUSSION.

The first and most important deduction to be made from these statistics is that pneumococcus pneumonia of Types I, II, and III has not occurred among the vaccinated troops, whereas twenty-six cases have occurred among the unvaccinated part of the camp. This is the best test of the value of the vaccination as a prophylactic measure. The one case of Type I pneumonia that developed 24 hours after the patient had received his first inoculation may properly be excluded from among the vaccinated cases, as the patient was probably already infected at the time he received the injection and could not have had time for the development of any appreciable immunity.

As Lister pointed out, the diminution or disappearance of certain types of pneumonia as the result of specific type inoculation is a more critical test of the efficacy of pneumonia prophylaxis than the mere simultaneous comparison of pneumonia rates in vaccinated and unvaccinated groups. It is true that the period of observation in this experiment has been short, but the immunity produced by the vaccine appears to have been adequate for this period of time. How much longer this immunity will last can only be determined by following these men and studying the cases of pneumonia that subsequently develop among them.

There have been only six cases of *Pneumococcus* Type IV pneumonia among the men who received two or more injections of the vaccine, while the control column shows thirty-three cases among the unvaccinated troops. The marked difference in these figures and the mild course which the *Pneumococcus* Type IV pneumonias ran in the vaccinated series might suggest that some cross-protection against Type IV pneumococcus has been afforded by the Type I, II, and III vaccine. This theory, however, is hardly admissible when we note that the same difference occurs in the incidence of streptococcus pneumonias

in the vaccinated and the unvaccinated troops. There were 106 cases of streptococcus pneumonia among the unvaccinated troops, whereas there were only seven streptococcus pneumonias among the vaccinated troops. Among the 3,500 colored troops, half the companies were vaccinated against pneumonia, the other half were not. There were twenty-eight cases of streptococcus pneumonia among the unvaccinated half and only two cases of streptococcus pneumonia among the vaccinated half, yet these men were living in the same part of the camp and closely associated on drill-grounds, and in recreation and amusement halls.

We have no explanation to offer for this difference. That an epidemic of streptococcus pneumonia occurred at about this time in the camp, there can be no question. Not only the bacteriological studies, but the clinical course, the frequency of empyema, the mortality rate, and the character of the autopsy findings confirm the bacteriological diagnosis. Why this epidemic should to a large extent have spared the vaccinated troops cannot be explained. The incidence of pneumonias, pneumococcic and streptococcic, was approximately equally distributed in the two groups of organizations previous to the beginning of the vaccination. While no explanation of this phenomenon can be offered, it presents no argument against the use of prophylactic vaccination against the pneumococcus, nor does it in the slightest degree weaken the importance of the fact that while twenty-six cases of Type I, II, or III pneumococcus pneumonia occurred among the unvaccinated, none occurred among the vaccinated troops. While it is still too early to draw final conclusions from this experiment, we feel that the results are sufficiently encouraging to justify further investigation along these lines.

SUMMARY.

1. From a study of the agglutinins and protective power of the serum of 42 persons vaccinated against the pneumococcus, Types I, II, and III, it is demonstrated that a definite immune response has been secured to Types I and II by the dose of vaccine employed. Little evidence of response to Type III can be demonstrated by these methods, but this is of less significance in that in animals it is rela-

tively difficult to secure antibodies against this strain in the serum, even though a considerable degree of active immunity may have been produced in the vaccinated animal.

2. The degree of response to the vaccination appears to be dependent upon the total dosage of each type of pneumococcus administered. While some response may be elicited by $2\frac{1}{2}$ billion cocci of each type, a much more constant and greater response follows 13 billion.

3. In subcutaneous administration the manner in which the total dosage is divided, whether given in a single large dose, in seven small daily doses, or in three to five moderate doses at 3 to 7 day intervals, seems to have little influence upon the degree of immune response, provided the total dosage is the same.

4. The local and general toxic reaction varies greatly in different individuals. The smaller the individual doses, the fewer are the severe reactions. This makes it desirable to divide the total dosage into as many inoculations as circumstances make practicable.

5. At Camp Upton 12,519 men have been vaccinated against *Pneumococcus* Types I, II, and III. Three or four doses were given at intervals of 5 to 7 days with a total dosage of 6 to 9 billion of Types I and II and $4\frac{1}{2}$ to 6 billion of Type III.

6. During the 10 weeks that have elapsed since the vaccination, no cases of pneumonia of these three types have occurred among the men who had received two or more injections of vaccine.

7. In a control of approximately 20,000 men there were twenty-six cases of *Pneumococcus* Types I, II, and III pneumonias during the same period.

8. The incidence of *Pneumococcus* Type IV pneumonia and streptococcus pneumonia was much less among the vaccinated troops than among the unvaccinated. No explanation has been advanced for this difference.

9. Small sterile infiltrations disappearing spontaneously occasionally follow the injection of large doses of pneumococcus vaccine and appear to be an expression of cutaneous hypersusceptibility.

10. The persons who develop these lesions exhibit local reactions to each dose of vaccine. They also give abnormally marked reactions to intradermal injections of pneumotoxin. They do not, however,

exhibit anything notable in the agglutinative or protective powers of their sera after vaccination. Whereas the immune response is characteristically specific for the type of pneumococcus, this reaction is not specific for any type. We have found no evidence that Type III is more prone to elicit these severe local reactions than are Types I and II.

11. Prophylactic vaccination against pneumococcus of Types I, II, and III is practical and apparently gives protection against pneumonia produced by these types. It remains to be determined how long this immunity persists.

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IS THE AMOUNT OF CALCIUM USUALLY GIVEN IN DILUTIONS OF COW'S MILK INJURIOUS TO INFANTS?

A REPLY TO THE ARTICLE ON "CALCIUM IN ITS RELATION TO THE
ABSORPTION OF FATTY ACIDS," BY BOSWORTH, BOWDITCH AND
GIBLIN, IN THE AMERICAN JOURNAL OF DISEASES OF
CHILDREN, JUNE, 1918.

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The claim is made by Bosworth, Bowditch and Giblin that by the use of a special "reconstructed" or "decalcified" milk, infants who are artificially fed will have a no greater excretion of fat in the feces than those fed at the breast. The position is taken that this special milk provides an improved form of feeding over simple cow's milk dilutions, because on the latter, children suffer a serious loss of fat through the excretion of soap stools; also that there follows from such feeding excessive constipation, leading frequently to a general upset, termed by the authors a "blowup."

We have had no opportunity to study Bosworth's decalcified milk and its effects; but we desire to present some conclusions drawn from results obtained in this laboratory which may perhaps allay the fears of calcium injury from the use of simple dilutions of cow's milk in infant feeding.

There are three questions suggested by this article which we desire to discuss:

1. Is there a serious loss of fat (otherwise than through diarrhea) when the usual simple dilutions of cow's milk are fed?

Of a group of thirty-two children from 2 to 15 months of age whom we have studied, eight received over 25 gm. of fat daily, the highest intake being 47 gm. None of the entire group had any tendency to diarrhea. Twenty-nine had a fat retention of 89 per cent.

or more of the intake, eighteen having over 90, and ten over 95 per cent. Only two of the whole group retained less than 80 per cent. of the intake, these retaining 78.9 and 78.2, respectively.

Table 3 in the article by Bosworth, Bowditch and Giblin, when analyzed, shows much lower percentages of fat retained than the foregoing values. Of the twenty-three cases in their table, only ten show a fat retention above 84 per cent., only two over 90 per cent. and none over 95 per cent., while in eleven the fat retention is less than 80 per cent. The difference between their results and ours is brought out in Table 1.

TABLE 1.

Comparison of Figures for Fat Retention.

	Bosworth et al.	Babies' Hospital Laboratory
Per cent. of total number with over 95 per cent. retention...	0.0	31.3
Over 90 per cent.....	8.7	56.3
Over 84 per cent.....	43.5	90.6
Under 80 per cent.....	47.8	6.2

We have not found such low percentages of fat retained as their figures show, except in cases of diarrhea, and occasionally in infants fed on protein milk. In the latter the intake of fat and the absolute retention are usually higher than with other milk preparations.¹

The extremely low fat retention shown in Table 3 of the article by Bosworth, Bowditch and Giblin raises the question whether in all their cases the excretion reported fairly corresponds to twenty-four hours' intake, or is excessively large because of a diminished excretion on the preceding day or two. This possibility occurs to us because of the peculiarity of the calcium excretion given in their Table 1. Computing from the formula used—fat 2 per cent., sugar 6 per cent., protein 1.6 per cent.—and the daily intake of 795 c.c., the total calcium intake could not have been more than 0.70 gm. of calcium oxid (CaO). The total calcium excretion reported is 1.697 gm., an excess over the intake of nearly 1.0 gm. of CaO. This we consider to be most improbable, being greater than we have ever found, even in the most severe

¹ The protein milk employed in the Babies' Hospital is made from whole milk, not from skimmed milk.

cases of rickets. Such excretion, however, might be accounted for, if the stools had been small, or if there had been none on the two preceding days. On this supposition the intake for three days would be approximately 2.1 gm., making, with the excretion reported, a positive balance of 0.4 gm., or about 0.13 gm. per day, which is a small retention. The daily fat excretion would then become about 1.84 gm., giving a retention of 14.06 gm., or 88.5 per cent. of the intake, a not unusually low retention for perfectly normal infants.

Bosworth and Bowditch claim that the high calcium content of the intake causes a serious loss of fat as soaps in the stools of bottle-fed infants. Accordingly, we present below some figures which may tend to reassure those who are likely to be alarmed over the "abnormality" of the large quantities of calcium soaps in the stools. In the group of thirty-two children just referred to, nineteen were receiving calcium to the extent of over a gram of CaO daily. Of these, only six had as much as 2 gm. of fat as soaps in the daily stools. All but one were utilizing over 85 per cent. of the ingested fat, while four absorbed more than 95 per cent. Table 2 shows the per cent. of fat retained and the amount of fat excreted as soaps by the children in this group who had the highest calcium intakes.

TABLE 2.

Fat Retention and Fat Excreted with High Calcium Intake.

Gm. CaO Intake	Per Cent. Fat Retained	Gm. Fat Excreted as Soap
1.80	95.8	0.78
1.66	89.4	1.68
1.50	93.2	1.12
1.47	87.7	0.79
1.41	87.1	1.76
1.39	95.5	0.99

A study of Table 2 shows that the maximum output, in the feces, of calcium bound as soaps was about 0.18 gm., since the combining weight of the higher fatty acids found in the stools is about ten times that of calcium oxid. Thus, it can be seen that the calcium which goes to form soaps is only a small part of the calcium intake.

Bosworth and Bowditch claim, however, that it is the degree of solubility of the calcium of the food which influences the formation of soaps. Using Bosworth's estimate of the "condition of calcium in cow's milk," which he gives in Table 6, the soluble calcium is seen to form 29.2 per cent. of the total calcium. In our entire series of thirty-two cases previously mentioned, the calcium which could possibly be held as soaps invariably falls below 25 per cent. of the intake. In the six with very high intake of calcium, the amount of calcium which could be held as soaps ranges from 7.0 to 12.5 per cent. of the intake, running well below half of Bosworth's estimate of the soluble calcium of the food. This proves conclusively that not all the soluble calcium is eliminated in the feces in the form of soaps. The inference that a high calcium intake causes a serious loss of fat does not seem to be warranted.

In a group of forty-six breast-fed infants, whose stools have been studied in this laboratory, twenty-five had over 40 per cent. of the fat excretion in the form of soaps. Thus, the presence of a considerable proportion of calcium soaps in the stools cannot be called an "abnormality," if breast-fed infants are to be taken as a standard. In one case, in which an exact record of the intake of breast milk was obtainable, the following results were found. With an intake of 41.9 gm. of fat, the total fat excretion was 2.26 gm., of which 63.8 per cent. was soap. The fat retention was 94.6 per cent. of the intake. The calcium intake was 0.355 gm. This child was at the time doing extremely well and has continued to do so. In this baby with a small calcium intake, practically the same results were found as with many bottle-fed infants with two or three times the calcium intake. This child had for its age (about 1 month) an unusually large fat intake.

It is conceded that breast-fed infants retain a high *percentage* of their fat intake and in many cases retain a large actual *amount* of fat. This may possibly be due to the biologic factors of the natural food rather than to any advantage in its chemical composition. Bosworth and Bowditch maintain that the soap excretion is dependent on the proportion of soluble calcium in the food, and on this ground explain the lower soap fat excretion when the food is breast milk. According to their Table 6, however, cow's milk, after being diluted with an equal volume of water, contains but little more soluble calcium than does

breast milk, that is, 0.0225 gm. of calcium in 100 c.c., in comparison with 0.021 in breast milk.

2. Is there danger of constipation with serious consequences in infants fed on simple dilutions of cow's milk?

Among the stools from the thirty-two children before mentioned, there was only one which from its appearance could be called constipated. The child was $7\frac{1}{2}$ months old and was doing well with an intake of 47.0 gm. of fat daily. The retention was 93.7 per cent. of the intake, the excretion of soap fat 2.3 gm. daily. The child has continued to gain and has done well without interruption up to the present. Two other children, 14 weeks and 8 months of age, respectively, were reported to be constipated. Though the stools of the latter were induced by a daily enema, the feces of both these children were soft and pasty and the daily amount small. They had, respectively, a retention of 96 and 97 per cent. of the intake, with daily soap fat excretion of 0.5 and 0.3 gm. This sort of constipation depends rather on the muscular tone of the bowel and on training than on the chemical composition of the stool. A tendency to constipation in infants receiving simple cow's milk dilutions is generally controlled without great difficulty. On the other hand, the desirability of formed or semi-formed soap stools in contrast to the ever dreaded tendency to diarrhea in bottle-fed infants, particularly in evidence in hospital patients, can hardly be overestimated. It is probably for this reason that protein milk with its high calcium and fat content plays so important a part in the control of diarrhea. Necessarily the formed stool has a large proportion of its fat in the form of calcium soaps, but, as has been previously emphasized, the total loss of fat in this way is very seldom, if ever, of serious consequence.

3. May there not be a danger in too low a calcium intake?

Unless the harm caused by a fairly high calcium intake can be quite definitely demonstrated, it would seem safer to allow an excess of calcium in the intake rather than to run any risk of providing less calcium than is needed for the normal growth of the bones. A number of cases of so-called intestinal infantilism have come under our observation. The most significant fact in the history of these cases is the extremely low intake of calcium in their previous feeding. The large calcium requirement of children with a tendency to rickets hardly

needs mentioning in this connection. The calcium in breast milk, though smaller in quantity, may be more available than that of cow's milk; however, it is well known that many cases of rickets and tetany occur in children fed too long exclusively on breast milk.

In conclusion, we believe it is yet to be demonstrated that infants fed on simple dilutions of cow's milk do not retain an adequate amount of fat, when the stools are formed or semiformed and soapy; nor do we think that a high calcium intake necessarily causes a large fat loss in the feces; while a great reduction of the calcium in the food of infants may be attended with considerable risk.

ALIMENTARY RENAL GLYCOSURIA.

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INTRODUCTION.

The glucose content of normal human urine is under 0.1 per cent., usually merely a trace. The kidneys normally excrete urine containing a lower concentration of glucose than is found in the blood. When the kidneys show a higher permeability for glucose, sugar should appear in the urine while the tissues still retain the normal power of utilizing dextrose. So-called "renal glycosuria" occurs on account of an abnormal permeability of the kidneys, without any disturbance of intermediary carbohydrate metabolism. In such a condition there are no diabetic symptoms and the blood sugar is normal.

The existence of this condition was first declared by Klemperer¹ at the Congress of Internal Medicine, in Berlin, 1896, and the conditions for its diagnosis were given as follows:

1. The glycosuria has almost no relation to the quantity of carbohydrate in the diet.
2. Not only is there no hyperglycemia, but sometimes even hypoglycemia.
3. When nephritis occurs the sugar in the urine diminishes or disappears.

Von Noorden,² in his book, "Zuckerkrankheit," refused to recognize the cases of renal diabetes previously reported, and held that the condition had not been demonstrated. Allen,³ who recognizes renal glycosuria, in his book, "Glycosuria and Diabetes," says that "the name diabetes is unjustifiable, diabetic symptoms are absent, but those

1. Klemperer, G.: Cong. Int. Med., Berlin, 1896. Ueber Regulatorische Glycosuria u. renalen Diabetes, Berl. klin. Wchnschr., 1896, 33, 571.

2. Von Noorden, C.: Die Zuckerkrankheit und ihre Behandlung, Ed. 6, Berlin, 1912, p. 37.

3. Allen, F. M.: Glycosuria and Diabetes. 1913.

of nephritis, neurasthenia and malnutrition are frequent;" and again, "almost always the glycosuria is very slight, a mere fraction of 1 per cent." Joslin⁴ recognized renal glycosuria and declared that an observation of several years was necessary for its diagnosis, clinical diagnosis without long observation of the patient being dangerous.

Since the initial report of Klemperer, a few reports of cases have been made in Germany by Lüthje,⁵ Bönninger,⁶ Weiland,⁷ Tachau,⁸ Frank,⁹ de Langen¹⁰ and Galambos;¹¹ and in America recently by Lewis and Mosenthal,¹² Murlin and Niles¹³ and Strouse.¹⁴ The details of these reports will be included in the chapter of discussion.

Report of Case.

As renal glycosuria is considered rare, observations on one case are here reported as follows:

History.—Man, aged 33, Japanese physician. No diabetic or neuropathic heredity; healthy childhood; at the age of 22 the patient had typhoid fever in the course of which albumin and casts were found in the urine for about two weeks. After university graduation in 1909, he was assistant at the university

4. Joslin, E. P.: Treatment of Diabetes Mellitus. 1917.

5. Lüthje, H.: Beitrag zur Frage des renalen Diabetes, München. med. Wchnschr., 1901, **38**, 1471.

6. Bönninger, M.: Beitrag zur Frage des Nierendiabetes, Deutsch. med. Wchnschr., 1908, **34**, 780.

7. Weiland, W.: Ueber einige ätiologisch bemerkenswerte Diabetesformen, Deutsch. Arch. f. klin. Med., 1911, **102**, 167.

8. Tachau, H.: Beitrag zum Studium des Nierendiabetes, Deutsch. Arch. f. klin. Med., 1911, **104**, 448.

9. Frank, E.: Ueber experimentelle u. klinische Glycosurien renalen Ursprungs, Arch. f. exper. Path. u. Pharmakol., 1913, **72**, 387 and 443.

10. de Langen, C. D.: Beitrag zur Kasuistik des renalen Diabetes, Berl. klin. Wchnschr., 1914, **51**, 1792.

11. Galambos, A.: Ueber den renalen Diabetes, Deutsch. med. Wchnschr., 1914, **40**, 1301.

12. Lewis, D. S., and Mosenthal, H. O.: Renal Diabetes, Bull. Johns Hopkins Hosp., 1916, **27**, 133.

13. Murlin, J. R., and Niles, W. L.: Renal Glycosuria, Am. Jour. Med. Sc., 1917, **153**, 79.

14. Strouse, S.: Renal Diabetes, Med. Clinics of Chicago, 1916, **2**, No. 2, p. 239.

hospital. He was healthy; no complaint; of good nutrition; body weight 50 kg.; height, 161 cm. Since 1911, his weight gradually increased to 54 kg.; there was increasing appetite, particularly for sweets; besides regular diet, cakes containing about 50 to 70 gm. of sugar were eaten once and sometimes twice in the afternoon. In April, 1913, after a meal consisting of 200 gm. rice, 150 gm. meat, vegetables cooked with sugar, after Japanese fashion, and much candy, an examination of the urine passed within two hours revealed 0.75 per cent. sugar. The determination of sugar in the urine was made qualitatively by the Almen-Nylander method, and quantitatively by Kumagawa and Suto's modification of Pavy's method. On the following day, after a lunch of 50 gm. bread and tea, the urine showed a slight sugar reaction. On the third day, after keeping in the meantime to carbohydrate-free diet, the urine showed no sugar reaction after a meal of 30 gm. bread; but after a lunch of 60 gm. bread, the glycosuria was present. He considered himself diabetic and treated himself accordingly. After eating 60 gm. bread at one time, sugar regularly appeared in the urine passed within two hours. The percentage of sugar was always below 0.7.

From June to November, 1913, a diabetic diet was followed. Sugar was excluded, but about 30 gm. carbohydrate in the form of bread and rice were taken with each meal, or from 90 to 100 gm. daily. The urine, examined from time to time showed an occasional sugar reaction. From January, 1914, the strict diabetic diet was remitted, but no sugar was taken; the diet included from 150 to 200 gm. carbohydrate daily, in the form of rice and vegetables. For a period of two years, the urine was not examined. Beginning April, 1916, after coming to America, sugar was added to the diet according to inclination, in the form of candy and ice cream. During this time the subject felt well and experienced no weakness. September 17, 1917, examination of the urine after the ordinary lunch showed almost 0.2 per cent. sugar. The determination of sugar in the urine was made qualitatively and quantitatively by Benedict's method.¹⁵ From September 20 to October 15, the diet consisted approximately of 100 gm. fat, 150 gm. carbohydrate, and from 150 to 200 gm. protein per day. Sugar was excluded; the urine showed no sugar reaction. But on a free diet containing sugar the urine two hours after meals occasionally showed a slight sugar reaction, not more than 0.2 per cent. The urine volume was not increased, and no sugar reaction was seen with mixed twenty-four-hour urine.

Experimental.—October 20, blood was taken at 9 a.m., before breakfast, after fasting over night, and determination of sugar in the blood made by the method of Lewis and Benedict;¹⁶ cholesterol in

15. Benedict, S. R.: The Detection and Estimation of Glucose in Urine, Jour. Am. Med. Assn., 1911, 57, 1193.

16. Lewis, R. C., and Benedict, S. R.: A Method for the Estimation of Sugar in Small Quantities of Blood, Jour. Biol. Chem., 1915, 20, 61.

whole blood by Bloor's method,¹⁷ and carbon dioxid in the plasma by Van Slyke's method.¹⁸ All showed normal. The blood pressure was also normal.

TABLE 1.

Findings in Plasma and Whole Blood Oct. 20, 1917, at 9 A. M., Before Breakfast, After Fasting Over Night.

Sugar in Plasma, Per Cent.	Cholesterol in Whole Blood, Gm. per 100 C.c.	CO ₂ Capacity of Plasma, Vol. Per Cent.
0.11	0.22	74

From November 29, every morning a certain amount of carbohydrate was ingested after the night's fast, with results as follows: After 33 gm. starch and 25 gm. fat, no sugar appeared in the urine, but with increase of starch to 39 gm., sugar appeared one or two hours after meals, but in quantities so slight that it was hard to estimate its percentage by Benedict's quantitative method. Blood sugar was examined for every thirty minutes after meals, but no hyperglycemia appeared. After ingestion of 150 gm. banana (34 gm. carbohydrate), the urine showed sugar, presumably because of the sugar content of the banana. After 50 gm. oatmeal (34 gm. carbohydrate), the urine showed sugar in two hours, but the blood sugar was normal. After 150 gm. potato (36 gm. carbohydrate), there was glycosuria in one hour, but no hyperglycemia. Therefore, after the ingestion of more than 34 gm. carbohydrate, the urine showed sugar after meals, without regard to the kind of starch taken, while the blood sugar was always normal.

In the third experiment (Table 3), large quantities of carbohydrate were ingested to determine the rise of blood sugar.

After the ingestion of 130 gm. rice (carbohydrate about 100 gm.), the urine showed from 0.16 to 0.29 per cent. sugar, which lasted to the fourth hour after meals. The total sugar excreted amounted to 0.25

17. Bloor, W. R.: The Determination of Cholesterol in Blood, *Jour. Biol. Chem.*, 1916, **24**, 227. The Distribution of the Lipoids (Fat) in Human Blood, *Jour. Biol. Chem.*, 1916, **25**, 577.

18. Van Slyke, D. D., Stillman, E., Cullen, G. E., and Fitz, R.: Studies of Acidosis, *Jour. Biol. Chem.*, 1917, **30**, No. 2, p. 289.

gm. Blood sugar, which reached its maximum in the first hour, amounted only to 0.115 per cent. No hyperglycemia was seen. After ingestion of 200 gm. starch, the urine showed from 0.2 to 0.3 per cent. sugar which lasted for 2.5 hours. The total amount of sugar excreted was 0.17 gm. Blood sugar reaching its maximum in 2.5 hours was 0.145 per cent. Sugar appeared in the urine after thirty minutes, when the blood sugar showed only 0.139 per cent.

In the fourth experiment (Table 4), glucose was taken in various quantities, in the morning as in the former experiments.

After 50 gm. glucose, sugar appeared in the urine in 30 minutes to 1.5 hours, the total amount being 0.07 gm. The maximum of blood sugar was 0.128 per cent., with return to normal at the end of the first hour. After 100 gm. glucose, sugar appeared in the urine at 30 minutes to 1.5 hours with a total amount of 0.9 gm.; blood sugar reached its maximum at 0.164 per cent. with return to normal within 3 hours. After 200 gm. glucose, sugar appeared after 30 minutes and lasted 4.5 hours, with a total excretion of 0.6 gm.; the maximum of blood sugar was 0.175 per cent. with return to normal at the third hour. Examination of the urine by polarimeter showed dextrorotation and agreed with the results obtained by Benedict's method. A fermentation test with yeast gave positive results, showing that the reducing substance in urine is glucose, not pentose. After the ingestion of 100 gm. levulose, the urine showed sugar after 30 minutes and continuing for 3 hours, with a total excretion of 0.09 gm. The polarimeter showed left rotation, blood sugar, a maximum of 0.143 per cent., with return to normal in the second hour.

Summary.—After the ingestion of more than 34 gm. starch, the urine showed sugar without any hyperglycemia; after 100 gm. starch the urine contained 0.25 gm. sugar, while after 200 gm. starch the urine showed only 0.17 gm. sugar; the maximum of blood sugar was 0.115 per cent. after ingestion of 100 gm. starch and 0.145 per cent. after 200 gm. starch. Furthermore, after the ingestion of 100 gm. glucose, more sugar was excreted in the urine than when 200 gm. were taken, while the blood sugar was higher and remained higher for a longer period after 200 gm. glucose was ingested than after 100 gm. When levulose was given it was excreted as levulose itself, not as glucose.

TABLE 2—*Determination of Carbohydrate To*

Date, 1917	Diet (Test meals at 9 a.m. after fasting over night)							Urine		
	Foodstuffs, Gm.					Carbo- hydrate, Gm.	Fat, Gm.	Volume, C.c.		
	Bread	Butter	Coffee	Cream	Salt			1 Hr.	2 Hrs.	3 Hrs.
Nov. 29	45	30	2 cups	27	25	53	40	24
Nov. 30	55	30	2 cups	..	1	33	25	45	39	21
Dec. 1	65	30	2 cups	..	1	39	25	46	28	25
Dec. 2	Banana 150	..	2 cups	33	..	44	40	23
Dec. 3	Oatmeal 50	30	2 cups	30	1	34	31	50	33	40
Dec. 4	Potato 150	30	2 cups	30	1	36	31	51	42	36

*Sugar test by Benedict's method, shows very slight reduction, qualitatively and meas

TABLE 3—*Starch Test. Tes*

Date, 1917	Diet (Test meals at 9 a.m. after fasting over night)							Urine					
	Foodstuffs, Gm.					Carbo- hydrate, Gm.	Fat, Gm.	Volume, c.c. Dextro					
		Butter	Coffee	Cream	Salt			30 Min.	1 Hr.	1.5 Hrs.	2 Hrs.	2.5 Hrs.	3 Hrs.
Dec. 5	Rice, 130 gm. (dry weight), boiled with 100 gm. water	30	2 cups	30	4	100	31	..	42	..	27	..	2
								..	+	..	+	..	+
Dec. 14	Starch (Merck), 200 gm. Paste made with 100 gm. water	..	2 cups	200	..	45	8	15	6	9	1
								+	+	+	+	+	-

als at 9 A. M., After Fasting Over Night.

Urine			Blood								Remarks
Dextrose			Sugar in Whole Blood, Per Cent.								
2 Hrs.	3 Hrs.	4 Hrs.	Before Test	30 Min.	1 Hr.	1.5 Hrs.	2 Hrs.	2.5 Hrs.	3 Hrs.	3.5 Hrs.	
—	—	—	Sugar in plasma in 1.5 hrs., 0.102 per cent.
—	—	—	
Trace*	—	—	0.081	0.085	0.087	0.082	0.085	0.086	0.071	
—	—	—	
Trace	—	—	0.071	
Trace	—	—	0.081	

M., After Fasting Over Night.

Urine.				Blood									
Hours, Per Cent. Quantities Sugar, Gm.			Excreted Sugar in Toto	Sugar in Whole Blood, Per Cent. Sugar in Plasma, Per Cent.									
2 Hrs.	3 Hrs.	4 Hrs.		Before Test	30 Min.	1 Hr.	1.5 Hrs.	2 Hrs.	2.5 Hrs.	3 Hrs.	3.5 Hrs.	4 Hrs.	5 Hrs.
0.26	0.29	0.18	= 0.25 gm. Polarimetric 0.19 per cent.	0.076	0.069	0.058	0.057	0.055
0.07	0.067	0.04		0.103	0.115	0.111	0.083	0.085
0.3	= 0.17 gm.	0.08	0.122	0.105	0.118	0.093
0.06		0.105	0.139	0.133	0.145	0.102

TABLE 4—*Sugar Test. Sugar at 9*

Date, 1917	Glucose Gm.	Water C.c.	Urine										Sugar in Hours, Per Total Quantities Sug		
			Volume, C.c. Dextrose										1 Hr.	2 Hrs.	3 Hrs.
			30 Min.	1 Hr.	1.5 Hrs.	2 Hrs.	2.5 Hrs.	3 Hrs.	3.5 Hrs.	4 Hrs.	4.5 Hrs.				
Dec. 19	50	300	30 +	15 +	16 ±	30 —	32 —	.. —	.. —	.. —	.. —	0.14 0.063	
Nov. 1	100	500	182 +	100 +	42 +	76 —	50 —	.. —	.. —	.. —	.. —	0.32 0.68	0.16 0.16	
Dec. 26	200	300	11 +	8 +	17 +	6 +	6 +	5 +	4 +	8 ±	1.26 0.24	1.08 0.19	0.99 0.12	
Dec. 8	Levulose 100	300	35 +	12 +	4 +	11 +	6 +	21 +	23 —	

The Renal Threshold for Glucose.—There are many reports concerning the renal threshold for glucose of the normal person. It is generally accepted that the kidney is permeable for sugar when the blood sugar reaches 0.16 to 0.17 per cent. Hamman and Hirschman¹⁹ say that in a normal individual the renal threshold is not a constant factor, but is usually between 0.17 and 0.18 per cent. of blood sugar, although some otherwise normal individuals have a low renal threshold, below 0.14 per cent. (0.12 to 0.14 per cent., or 0.13 to 0.14 per cent.). Foster²⁰ found the renal threshold between 0.149 and 0.164 per cent. in a patient who had undergone ether narcosis. Jakobsen²¹ gives the blood concentration as 0.16 to 0.17 per cent. In the person

19. Hamman, L., and Hirschman, I. I.: Studies on Blood Sugar, *THE ARCHIVES INT. MED.*, 1917, **20**, 761.

20. Foster: *Am. Soc. Adv. Chem. Invest.*, 1917. Cited by Joslin (Footnote 4).

21. Jacobsen, Aage Th. B.: Untersuchungen über den Einfluss verschiedener Nahrungsmittel auf den Blutzucker bei normalen, zuckerkranken u. graviden Personen, *Biochem. Ztschr.*, 1913, **56**, 471.

Fasting Over Night.

Total Sugar Excreted	Blood								
	Sugar in Whole Blood, Per Cent. Sugar in Plasma, Per Cent.								
	Before Test	30 Min.	1 Hr.	1.5 Hrs.	2 Hrs.	2.5 Hrs.	3 Hrs.	3.5 Hrs.	4 Hrs.
7 gm.	0.082	0.085	0.095	0.088
	0.097	0.128	0.116	0.094
gm.	Plasma
	0.09	0.164	0.135	0.123	0.131	0.095
gm.	0.081	0.112	0.099	0.098	0.082	0.097
2 per cent. in toto	0.091	0.175	0.133	0.121	0.105	0.133
rimetric 0.83 per cent.									
gm.									
per cent.									
metric left rotation	0.072	0.071	0.061	0.094	0.061
per cent.	0.102	0.143	0.128	0.106	0.114

reported on here, the renal threshold is as follows: whole blood, 0.069 to 0.071 to 0.081 to 0.085 per cent.; plasma, 0.115 per cent.

Hyperglycemia After the Intake of Carbohydrate and Glucose.—Much has been written on this subject, but very little that is trustworthy. For this the apparent reason is that prior to the introduction of Bang's method in 1914 and the present more accurate method of Lewis and Benedict a determination involved the use of much more blood than is now required, with consequently but one or two tests in each case, whereas an examination is requisite every thirty minutes. Consequently the conclusions drawn from former experiments involve confusion.

Frank,²² in 1911, asserted that in eight persons hyperglycemia of from 0.12 to 0.18 per cent. was found in the blood plasma after the intake of 100 gm. glucose in one hour's time, and that three persons showed a little sugar

22. Frank, E.: Weitere Beiträge zur Physiologie des Blutzuckers, *Ztschr. f. physiol. Chem.*, 1911, **70**, 291.

in the urine. Tachau²³ (1911), reported that no hyperglycemia was seen in one hour's time after a dose of 100 gm. sugar. Bing and B. Jakobsen²⁴ (Bang's micromethod) found hyperglycemia of 0.098 to 0.17 per cent. Among ten persons, after intake of glucose, two showed 50 per cent. and one showed 70 per cent. rise of blood sugar. The average rise after one hour was 36 per cent., and after two hours, only 11 per cent. These authors believe that a rise of more than 50 per cent. at the end of the first hour must be considered as pathologic.

Th. B. Jacobsen²¹ (Bang's micromethod) found that 100 gm. glucose produces a rapid hyperglycemia which usually reaches its maximum in from fifteen to thirty minutes after the intake, keeps up one to three hours, and then falls to normal or even below. His experiments included fourteen persons whom he held to be normal. Among them six persons showed no urinary sugar, with blood sugar reaching from 0.12 to 0.16 per cent. Another group of eight persons showed sugar in the urine, with blood sugar of 0.17 to 0.227 per cent. However, the greatest amount excreted was only 1.38 gm. In the first group of six persons showing sugar, the hyperglycemia lasted about 1.75 hours; in the second group, about 2.5 hours. After 167 gm. bread (100 gm. carbohydrate), all fourteen persons reacted with a hyperglycemia (0.138 to 0.206 per cent.) which lasted from two to four hours, and six cases showed glycosuria (0.06 to 0.92 gm.). The only difference between the effect of dextrose and of starch was in the rapidity of the rise and fall of the blood sugar curve; that is, only a quantitative difference due to the difference in the rate of absorption. Jacobsen's results seem to be somewhat higher than normal, according to our present conception. There is a possibility that the use of the method was not accurate, with consequent high results, or that the experiments included persons of carbohydrate tolerance lower than normal.

Hopkins²⁵ (Bang's micromethod), in experiments with eight persons, found that the blood sugar reached its maximum (0.14 to 0.156 per cent.) in 30 minutes and lasted 2 hours. No urine examinations are reported. Graham²⁶ (Bang's micromethod), with three persons, noted that after 100 gm. glucose, the greatest rise of blood sugar was from 0.09 to 0.18 per cent., and the smallest was from 0.095 to 0.14 per cent. The maximum was usually reached in 20 minutes with return to the original level in from 1 to 1.5 hours. Cummings and

23. Tachau, H.: Ueber alimentäre Hyperglykämie, *Deutsch. Arch. f. klin. Med.*, 1911, **104**, 432.

24. Bing, H. J., and Jakobsen, B.: Blutzuckeruntersuchungen unter normalen u. einigen pathologischen Verhältnissen, *Deutsch. Arch. f. klin. Med.*, 1914, **113**, 571.

25. Hopkins, A. R.: Studies in the Concentration of Blood Sugar in Health and Disease as Determined by Bang's Micromethod, *Am. Jour. Med. Sc.*, 1915, **149**, 254.

26. Graham, G.: Variations in the Blood Sugar in Health, *Jour. Physiol.*, 1915-1916, **1**, 285.

Piness,²⁷ after the intake of 100 gm. glucose, found about a 50 per cent. rise during the first hour, with a drop of about one half of this during the second hour. In subjects having a low tolerance for sugar, the rise following the ingestion of 100 gm. glucose is distinctly higher than normal and the high level is well sustained up to the end of the second hour.

Hamman and Hirschman¹⁹ (Lewis and Benedict's method) say that in the normal person, after the ingestion of 100 gm. glucose, the blood sugar rises promptly to a level not exceeding 0.15 per cent., the high point is usually reached in about 30 minutes, and the whole reaction lasts from 1 to 2 hours, occasionally longer. They noted in many instances that where there is a considerable glycosuria, the excretion of sugar continues long after the blood sugar has fallen below the level at which it first appeared.

This latter fact—the continued excretion of sugar after the fall of blood sugar—may also be remarked in my case. Here, the maximum height and duration of the hyperglycemia after starch and glucose remain within the normal limit; the excreted sugar is below 1 gm., and there is no proportion between the glucose intake and the excreted sugar. Therefore, it is difficult to consider this case as a diabetes mellitus, but there is almost no doubt that it is a renal glycosuria, since the sugar excretion is due to the abnormally lowered threshold of the kidney, not to a pathologic hyperglycemia.

The Blood Sugar and the Renal Threshold of Diabetes.—The blood sugar of the diabetic is in higher concentration than normal. It is only in the mild form of the disease or under strict treatment that the blood sugar is normal. Following the administration of 100 gm. of glucose the rise in the blood sugar is greater than normal, and the rise is sustained longer than in normal men. Jacobsen²¹ found in two cases of diabetes that an appreciable amount of sugar appeared in the urine, with sugar-freedom at the beginning of the experiment, and blood sugar below 0.15 per cent. He explained these facts on the ground that the diabetic excretes more sugar than normal at a certain level of blood sugar. It is also known that there is frequently a higher threshold for sugar in diabetics without evident renal lesions; some are free from glycosuria even when the blood sugar reaches 0.5 per cent.

27. Cummings, R., and Piness, G.: A Study of Blood Sugar. Comparison of Tolerance for Glucose in Diabetic and Normal Subjects, *THE ARCHIVES INT. MED.*, 1917, 19, 777.

According to Cummings and Piness,²⁷ in the mild form of diabetes, following the administration of 100 gm. glucose, the rise of blood sugar is longer sustained than normal, even to the end of the second hour. In a really or moderately severe case the blood sugar is higher two hours after intake of 100 gm. glucose than one hour after. Hamman and Hirschman¹⁹ confirmed the finding that the hyperglycemia in mild diabetes is abnormal in definite respects: the blood sugar rose more slowly and reached its highest point later than normal; it also rose to a much higher level, all their three cases exceeding 0.2 per cent. Likewise, the fall of the blood sugar occurred much more slowly than in the normal, the whole reaction occupying from three to four hours. These authors believe that the *duration* of the hyperglycemia is a more important index of the severity of the alteration of carbohydrate metabolism than is the height of the hyperglycemia. Concerning the renal threshold in diabetes, they found that mild cases show a normal level, while moderately severe cases sometimes show a threshold below 0.15 per cent., but usually higher.

Graham,²⁸ in 1916, reported lower and higher thresholds for diabetes. His patient of lower threshold—a man of 32—in whom glycosuria had been detected five years previously, followed an unrestricted diet most of the time, with a sugar output of about 10 to 30 gm. daily. The blood sugar before a meal and after semi-starvation for twenty-four hours varied from 0.1 to 0.13 per cent., and sugar was always present in the urine. After 100 gm. dextrose the blood sugar rose 0.22 per cent. in one hour and had not quite fallen to its original level three hours later. The results in this case seem to indicate that the escape of sugar was due in some degree to permeability of the kidneys, or in other words, this case might represent a combination of renal glycosuria and true diabetes.

The Test of the Renal Function.—As it was clear that my case showed a lowered threshold for sugar, examinations of the renal function were made. McLean's²⁹ adaptation of the Ambard constant was employed for the determination of the excretion of urea and sodium chlorid.

28. Graham, G.: Variations in the "Leak Point" in Diabetes Mellitus. I. A Low Level, Jour. Physiol., 1915, **49**, Proc. Physiol. Soc., p. 47.

29. McLean, F. C.: The Numerical Laws Governing the Rate of Excretion of Urea and Chlorids in Man, Jour. Exper. Med., 1915, **22**, Nos. 2 and 3, pp. 212 and 366.

TABLE 5.

The Relation of the Rate of Urea Excretion to Concentration in the Blood.

Dec. 21, 1917 (10:20 a. m. to 11:32 a. m.); bleeding at 10:50; total urine, 168 c.c.

Weight, Kg.	Urea				NH ₃	Acidity (Folin)
	Per Liter of Blood, Gm.	Per Liter of Urine, Gm.	Per 24 Hours, Gm.	Index	Per Liter of Urine, Gm.	N Acid per 10 Liter of Urine, C.c.
51	0.244	9.05	30.41	270	0.229	55.8

Breakfast at 8 a. m.; bread, 40 gm.; butter, 20 gm.; 2 eggs; half grape fruit; 1 cup of tea.

TABLE 6.

The Relation of the Rate of Chlorid Excretion (Calculated as Sodium Chlorid) to Concentration in Plasma.

Same morning.

Weight, Kg.	Sodium Chlorid					
	Per Liter of Urine, Gm.	Per 24 Hours, Gm.	Per Liter of Plasma			Threshold
			Calculated, Gm.	Actual, Gm.	Difference, Gm.	
51	7.8	26.2	6.21	6.25	+0.04	5.66

McLean has shown that the urea excretion index as an indicator of renal function is preferable to determinations of blood urea interpreted without reference to urea excretion. The tests were conducted as follows: breakfast at 8 a.m.; at 9:50, 200 gm. water drunk; at 10:20, urine passed; at 10:50, blood taken; at 11:32, that is, seventy-two minutes after the bladder was first emptied, the urine was collected, measured, and used for analysis. McLean's tables show that the normal concentration of urea in the blood varies from 0.2 to 0.5 gm. per liter. The normal urea index lies above 80, with an average of 120, based on 100 tests. Any index below 80 is considered abnormal, and the degree of impairment of functional ability or damage to the kidneys becomes greater as the index gets lower. An index above 300 may occur in healthy young persons with low blood urea; it may result from the washing out of urea with a high fluid output, or it may occur with "vascular hypersensitiveness" (Schlayer).

The urea index of my case is 270. This is due to the high fluid output, the rate of water excretion for twenty-four hours being 3,300

c.c. by calculation, while the daily volume of urine is always from 1,200 to 1,800 c.c. actually.

McLean has confirmed Ambard and Weill as to the law of chlorid excretion in relation to its blood concentration, and has found that normally the plasma chlorid varies from 5.62 to 6.25 gm. per liter according to the amount of salt ingested. There is a close agreement between the chlorid calculated in the plasma by Ambard and Weill's constant and that actually found.

The actual plasma sodium chlorid content in my case is 6.25, and the difference between the actual content and the calculated result is 0.04. Consequently, in this case the renal test shows no disturbance of kidney function according to the urea and chlorid excretion.

The Relation Between Diabetes and Nephritis.—It is well known clinically that when chronic nephritis occurs as a complication in diabetes, the kidney excretes less sugar than before and the sugar content in the urine is diminished. It is also known that hyperglycemia (without glycosuria) often occurs in cases of nephritis.

Myers and Bailey³⁰ lately reported hyperglycemia in nephritis and discussed the elevating influence of the nephritis generally accompanying advanced diabetes on the threshold of sugar excretion. According to Hopkins,²⁵ nephritic cases all furnish very high figures of blood sugar after feeding glucose, the duration of hyperglycemia lying between that of normal and of diabetic patients. Bing and B. Jakobsen,²⁴ in some cases of nephritis, after ingestion of glucose found an abnormal hyperglycemia but no alimentary glycosuria. Hamman and Hirschman¹⁹ say that the renal threshold in nephritis is often above 0.2 per cent. In spite of the fact of the frequently high renal threshold in nephritis, however, there is sometimes a normal threshold in nephritis, and these authors found also a nephritis with a low threshold for glucose, in the neighborhood of 0.15 per cent.

As regards the kidney function in diabetes, Fitz³¹ found that the urea index in the majority of the cases tended to be normal or abnor-

30. Myers, V. C., and Bailey, C. V.: The Lewis and Benedict Methods for the Estimation of Blood Sugar, with Some Observations Obtained in Disease, *Jour. Biol. Chem.*, 1916, **24**, 147.

31. Fitz, R.: Observations on Kidney Function in Diabetes Mellitus, *THE ARCHIVES INT. MED.*, 1917, **20**, 809.

mally high. In diabetes the plasma chlorid is usually lower than would be calculated from the chlorid excretion according to the formula of Ambard and Weill. McLean²⁹ found a lowering of the plasma chlorid in the majority of his observations in twenty-four cases of diabetes.

Review of the Hitherto Reported Cases of Renal Glycosuria.

A search of the literature of renal glycosuria gives the following data:

1. Klemperer¹ (1896, Congress of Internal Medicine, Berlin) reported a case of old nephritis which showed 0.35 per cent. sugar in the urine with the normal figure for blood sugar; the intake of bread and 150 gm. glucose exerted no influence on the sugar content either of urine or blood; sugar in the whole blood was 0.175 per cent., which, according to our modern conception, must be considered as hyperglycemia. At the Congress of Internal Medicine in Wiesbaden in 1913 where the problem of renal glycosuria was again discussed, Klemperer's case was no longer recognized as a renal glycosuria.

2. Lüthje⁵ reported a patient, aged 22, a merchant, with gonorrhea, cystitis, and probably ascending pyelonephritis. In the urine were found albumin and sugar below 1 per cent., reaching at the utmost 15 gm. a day. There was a constant excretion of sugar showing but very slight variation in the quantity despite great difference of carbohydrate intake per day. The single determination of sugar in the blood showed sugar as 0.055 per cent. in the whole blood. No mention was made, however, of the time or the relation to the diet, so that a conclusion concerning this case is consequently impossible. Lüthje claimed a relation between glycosuria and the nephritis because his case was apparently free from sugar *before* the nephritis, while sugar appeared in the urine shortly *after* the nephritis.

3. Bönninger⁶ describes a patient, a man, aged 37, an alcoholic, who said that he had once shown urinary sugar of 2 per cent. After admission to the hospital he excreted sugar to the extent 0.2 per cent. on normal diet. During thirty-three days' observation in hospital, the percentage of sugar in the urine was remarkably constant in spite of great differences in carbohydrate intake, varying only between 0.1 and 0.5 per cent. The sugar content of the serum was 0.097 per cent.; of whole blood, 0.078 per cent. Occasionally when the urine contained 0.5 per cent. sugar, the blood sugar was 0.062 per cent. This patient, who was observed for six years, is in perfect health and still excretes a small amount of sugar. His son is also affected with renal glycosuria.

4. Weiland⁷ makes a report of three cases which are not all, however, renal glycosuria.

5. Tachau⁸ reports a patient, 21 years of age, a merchant, who developed gastro-enteritis after an excessive indulgence in fruit. The urine showed albumin, erythrocytes and casts (transient acute nephritis). The following schedule shows the results of experiments.

Diet	Days Observed	Urinary Glucose in 24 Hours, Gm.
Carbohydrate-free.....	4	0
Mixed (158 to 192 gm. C. H.).....	6	0
Mixed (124 to 412 gm. C. H.).....	15	1.1 to 6.0
Mixed plus 100 gm. dextrose.....	3	0.6 to 4.2

The percentage of sugar in the whole blood after fasting was from 0.085 to 0.086; one hour after 100 gm. glucose it was 0.07 to 0.109.

6. E. Frank⁹ reports two cases: (a) a patient aged 51, who for four years had suffered from severe diarrhea of four weeks' duration, four or five times a year, without apparent cause. After the attacks glycosuria lasting about ten days was observed. Dextrose in the urine ranged from 0.05 to 0.5 per cent.; sugar in the whole blood equaled 0.09 per cent.; in the plasma, 0.106 per cent.; (b) this patient suffered from a nervous complaint. Sugar in the urine was from 0.4 to 0.65 per cent.; in the whole blood, 0.08 per cent.; in the plasma, 0.08 per cent. In both cases, however, the time of the blood sugar determination and its relation to the diet were not shown, consequently, this report is incomplete. Frank's paper is valuable rather as a report of the literature and of his own experiments of renal glycosuria in animals under heavy-metal intoxication, as mercury, uranium and chrome. In these experiments he proved a true renal glycosuria. His exposition of increased renal permeability after poisoning with heavy metals, and also during pregnancy, suggests the existence of a clinically permanent renal glycosuria. Frank further emphasized the importance of estimating sugar in the plasma instead of in the whole blood.

7. In de Langen's¹⁰ case, a patient of 22 years, sugar was always present in the urine, the quantity of excreted sugar varying between 4 and 19 gm. in twenty-four hours, and showing no proportional relations to the quantity of carbohydrate intake. After a carbohydrate-free diet there was a high content of sugar in the urine, while the smallest excretion of sugar was seen after a high carbohydrate intake. Intake of theobromin sodium salicylate (diuretin), which, according to some authors, influences sugar excretion, had no influence in this case. Blood sugar was determined in whole blood by Bang's method; there was hypoglycemia (0.054 to 0.073 per cent.); these results are, however, not decisive because the determination was not made until three hours after the meals instead of one or two hours, but they probably serve to rule out diabetes.

8. Galambos¹¹ reported a patient 50 years of age. During observation over a period of twenty-one days, while the carbohydrate content of the diet was

between 50 and 354 gm. per day, the glycosuria varied between 66 and 198 gm. and the concentration of urinary dextrose between 2.6 and 7.4 per cent. Blood sugar during fasting showed 0.089 and 0.052 per cent.; one and a half hours after taking 100 gm. dextrose it was 0.17 per cent. After caffeine, the urinary sugar was increased with the increase of urine volume. This case shows polyuria, polydipsia and acidosis, with much urinary sugar. The respiratory quotient was said to be 0.709 in fasting.

9. Lewis and Mosenthal¹² report the case of a man aged 29, who for the previous two or three years had had a tendency to increased frequency of urination during the day, but not at night. This condition had evidently been a pollakiuria rather than a polyuria, as the quantities voided apparently had not exceeded the normal. There were no diabetic symptoms. The patient excreted from 11 to 13 gm. sugar in twenty-four hours on "ward light diet." After the ingestion of 100 gm. glucose a hyperglycemia of 0.15 per cent. was evident within thirty-five minutes. Two hours and five minutes after the experiment was begun the blood sugar level returned to normal and subsequently became depressed below the normal level. The phenolsulphonephthalein test showed an excretion of 42 per cent. in two hours. Ambard's constant, determined at various times, was from 0.07 to 0.11 per cent. The kidneys were intact as far as physical and urinary signs were concerned; functional tests, however, revealed some impairment, as shown by a slightly diminished phenolsulphonephthalein excretion and an Ambard's constant barely within the upper normal figure.

10. Murlin and Niles¹³ report a case of a man aged 20, with no diabetic heredity, who two years previously had furuncles, polyuria and thirst with loss of 10 pounds in one month. After admission to the hospital, sugar in the urine was between 19 and 34 gm. when the carbohydrate in the diet was between 15 and 100 gm. Examination for blood sugar at 11 a. m., five hours after breakfast, revealed a normal percentage on several days. As this case was accompanied by diabetic symptoms, however, and as the blood sugar was not tested at the proper time, it is hard to classify it as renal glycosuria.

11. Strouse¹⁴ had a patient aged 13 who felt perfectly well. With noncarbohydrate diet the urine showed a trace of sugar, not quantitatively measurable. After prolonged ingestion of carbohydrate, the urine showed the same trace of sugar, apparently no more and no less than was present when the patient was on a carbohydrate-free diet. Blood sugar was 0.04 per cent. one hour after a meal rich in carbohydrate.

DISCUSSION.

There has been a gradual change from the former to our present conception of renal glycosuria. Klemperer¹ regarded this condition as a sort of anomaly of metabolism characterized by normal content of

blood sugar with independence of sugar excretion from carbohydrate intake. Lüthje⁵ and Naunyn³² believed that a relation existed between renal glycosuria and nephritis. Naunyn³² remarked a renal glycosuria occurring with granular nephritis; he reported three cases, but the lack of blood sugar determination leaves us uncertain whether there was hyperglycemia or hypoglycemia. Naunyn believes that nephritis influences the epithelia of the kidney to facilitate the excretion of sugar. This same author also reported glycosuria occurring after a renal hemorrhage which had no apparent relation to the diet and which disappeared without any restriction of diet.

It is believed that glycosuria in pregnancy and in the puerperium is probably a renal process. Furthermore, Frank's⁹ experiments confirm the work of many investigators concerning experimental renal glycosuria due to an increased permeability for sugar after poisoning with mercury, uranium and chromate. Among the clinical cases of renal glycosuria already cited, the ones showing a previous affection of the kidney are those of Klemperer, Lüthje and Tachau. My patient also had a record of febrile albuminuria during typhoid fever ten years previously. But the general conception now is that the existence of kidney affection, that is, anatomic change of the kidney (morbus brightii and contracted kidney) is not necessary for the occurrence of renal glycosuria. The reasons for the permeability of the kidney to sugar are not yet known.

It has been shown that the blood sugar rises after meals (Bing and B. Jakobsen,²⁴ Th. B. Jakobsen,²¹ Strouse³³), and Joslin⁴ says that the percentage of sugar in the blood of normal individuals rises promptly after meals and may reach 0.17 per cent. Accordingly, if the sugar threshold of the kidney is below the normal level, 0.17 to 0.18 per cent., there must occur regularly after meals a glycosuria which lasts for a certain time and disappears spontaneously. It is pointed out by Hamman and Hirschman¹⁹ that the lowered renal threshold may occur in otherwise normal individuals. They believe that a relation between the lowered renal threshold and renal glycosuria is obvious.

32. Naunyn, B.: *Der Diabetes mellitus*, Wien, 1906, p. 136.

33. Strouse, S., Stein, I. F., and Wiseley, A.: *The Accurate Clinical Study of Blood Sugar*, Bull. Johns Hopkins Hosp., 1915, 26, 211.

The intensity of the renal glycosuria will vary with the degree of the affection of the kidney, that is, its permeability to dextrose: (1) If the permeability is severely affected, with marked depression, urinary sugar will constantly appear even when the blood sugar content is very low. (2) When the renal permeability is but mildly affected, sugar will appear only at the high level of hyperglycemia of physiologic limit, and there will be no sugar in the urine with carbohydrate-free diet, because with that diet there occurs no high physiologic hyperglycemia. Under a diet rich in carbohydrate, the blood sugar will rise to the high level of physiologic hyperglycemia and sugar will appear in the urine. This high level of physiologic hyperglycemia, is, in the normal subject with normal permeability of the kidney, a preglycosuric level.

Since we know that the blood sugar undergoes marked and rapid fluctuation after the ingestion of carbohydrate, and that urinary sugar has some relationship to hyperglycemia after meals, it is hardly possible to deny, to a certain extent, at least, the existence of a relationship of urinary sugar in renal glycosuria to the carbohydrate content of the diet. In the case under consideration there was no urinary sugar when the carbohydrate in the diet was below 34 gm., because the elementary hyperglycemia occurring with that diet is under the threshold of the kidney for sugar. Blood sugar was highest after 200 gm. glucose, but the urinary glucose in that case was less than after 100 gm. glucose.

From the foregoing facts it is apparent that renal glycosuria may depend to a certain extent on the carbohydrate content of the diet. Therefore, a classification of this condition is necessary: (1) When the renal permeability is but slightly affected, glycosuria may not appear after a carbohydrate-poor diet; but, when it is severely affected, glycosuria will not disappear even after the entire withholding of carbohydrate from the diet. (2) Urinary sugar is not excreted proportionately to the increase and decrease of carbohydrate in the diet.

Von Noorden² remarks that in mild diabetes the urinary sugar is often independent of the carbohydrate intake. Therefore, too much attention must not be paid to the fact of the independence of urinary sugar to the carbohydrate in the diet.

The most important factor in the diagnosis of renal glycosuria is the presence of the normal level of the blood sugar when sugar appears in the urine.

Von Noorden² also says that renal glycosuria must be observed not a few weeks or months, but over a long period of time, since its progress is different from that of ordinary diabetes. Joslin⁴ remarks the same thing. The author's case has been observed for five years, and the tolerance for carbohydrate is still the same and the subject is in good health.

Suggestions as to the Nature of Renal Glycosuria.—In the foregoing discussion I have tried to show that renal glycosuria may be defined according to the following characteristics:

1. Glycosuria occurs without *abnormal* hyperglycemia. Abnormal hyperglycemia, as previously stated, is entirely different from alimentary hyperglycemia of from 0.16 to 0.17 per cent., which is physiologic. Glycosuria occurs although the blood sugar is within this physiologic limit.

2. There is no disturbance of carbohydrate metabolism; the nature of renal glycosuria is entirely different from that of true diabetes mellitus, and consequently, there are clinically no diabetic symptoms.

3. Urinary glucose may or may not have some relationship to the carbohydrate in the diet, according to the degree of permeability of the kidney. In a mild case, sugar will disappear from the urine during a carbohydrate-free diet; glucose usually appears after rich carbohydrate intake.

4. During a long period of time no progressive increase of the abnormality occurs.

It is obvious that the term "renal diabetes" is not fitting for the characteristics just described, since the condition is not diabetes, but is "alimentary renal glycosuria."

Diagnosis of Renal Glycosuria.—Notwithstanding the fact that it is perfectly simple to observe that the lowering of the renal threshold results in the appearance of glucose in the urine without disturbance of intermediary carbohydrate metabolism, nevertheless, the question of renal glycosuria was left for a long time unsolved. The recent improved methods of blood sugar analysis have afforded the first opportunity of settling the question of blood sugar and especially of

alimentary hyperglycemia. Now we know that when an individual shows sugar in the urine we must determine the blood sugar before we can make the diagnosis "diabetes," and furthermore, that in case of doubt, sugar must be determined in the plasma, because the renal threshold pertains most strictly to it. Examination of blood sugar is required not only once after fasting, but also at least twice every hour after a certain carbohydrate diet with resultant glycosuria. The elevation of the blood sugar and the renal threshold of the individual must also be studied. Comparison of single blood sugar determinations is without value. By these means, just stated, we can learn whether the glycosuria is due to disturbance of carbohydrate metabolism or to the increased permeability of the kidney for sugar, according as the hyperglycemia present is within the physiologic limit or not. We learn at the same time the level of the renal threshold in the patient.

The glucose test, which must next be considered, consists in the study of the blood and urine of the patient beginning thirty minutes and continuing two or three hours following the intake of 100 gm. glucose with from 250 to 300 gm. water, after an over-night fast. This test shows the condition of carbohydrate assimilation of the individual. If the hyperglycemia still remains within the physiologic limit after the test, we may exclude diabetes mellitus and consider that the urinary sugar is due to the increased permeability of the kidney to sugar.

The one more important point is the observation of the case over a period of years, because, as remarked by von Noorden, there is a form of mild diabetes which shows a high tolerance for sugar in the early period of the disease. To differentiate renal glycosuria from this mild form of diabetes mellitus one must determine the course and watch the outcome.

Since renal glycosuria brings about no injury to the health or annoyance to the individual, it is possible that persons with a lowered sugar threshold may unconsciously for a long period excrete sugar in the urine after meals. The absence of symptoms may quite possibly conceal many cases of renal glycosuria which are not at present credited. It is also possible that some of the cases of the so-called mild diabetes in practice may belong to this form of gly-

cosuria because the majority of them are diagnosed only by the examination of sugar in the urine, or, at most, by a single determination of blood sugar without relation to the preceding meal. An imperfect examination might easily diagnose as mild diabetes cases which in reality are renal glycosuria with a lowered threshold of the kidney for sugar and perfect carbohydrate metabolism.

Prognosis of Renal Glycosuria.—The present conception of renal glycosuria is that this condition is stationary; the glycosuria has no tendency to increase and the subject remains in good health. My case shows, during five years, only the same slightly depressed threshold and consequently only intermittent glycosuria after a meal rich in carbohydrate. Whether this condition will continue for a long period of time, say 10 or 20 years, or whether sooner or later a marked depression with constant excretion of sugar in the urine will occur, only the future can reveal.

Therapy of Renal Glycosuria.—It is believed at present that it is useless to keep to a strict diet in renal glycosuria. However, with the cases hitherto reported so few and the time of their observation so short, there is no surety that it is harmless to give carbohydrate and glucose in large amounts to persons with lowered kidney thresholds, since there is a chance that the depression might be thereby increased. The question is still open.

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PROTEOSE INTOXICATIONS AND INJURY OF BODY PROTEIN.

III. TOXIC PROTEIN CATABOLISM AND ITS INFLUENCE UPON THE NON-PROTEIN NITROGEN PARTITION OF THE BLOOD.

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This paper contains experimental data which supplement the published experiments of Whipple, Cooke, and Stearns (1, 2) who showed the profound influence of proteose injections upon the elimination of nitrogen in the urine. A single injection of toxic proteose in a fasting dog will cause a great rise in the base-line level of nitrogen elimination—often an increase of 4 to 6 gm. above normal, lasting many days. This, of course, indicates a great destruction of body protein following a single intravenous toxic injection—an acceleration of tissue autolysis lasting over 2 to 7 days with a maximum nitrogen elimination occurring during the second 24 hour period. The toxic proteose causes a disturbance of tissue equilibrium which is not restored to normal for several days following a severe intoxication. It has been suggested that the toxic proteose may so injure cell protoplasm that the resultant cell autolysis may form other toxic split products capable of further injury to the body—a true vicious circle of intoxication (1). For this reason it seemed desirable to study the blood by all available methods to determine the nitrogenous products of increased tissue autolysis *in vivo*.

The term "proteose intoxication" is used in a liberal sense in this communication. It is generally admitted that the chemistry of the proteoses is at best unsatisfactory. There are few proteoses which can be accepted as chemically pure by a critical chemist and very few indeed that will meet the requirements for pure proteoses demanded

by Gibson (3). It must be admitted that even the pure proteose preparations may be mixtures of several proteoses. It is claimed that these pure proteoses are relatively inert, but we believe it is safer to say that the complicated methods used to purify the proteoses will completely denature these several proteoses. Repeated precipitation of the proteoses used in our experiments by means of alcohol will completely remove all toxicity, yet this does not mean a removal of toxic impurities, because a collection of all the alcoholic filtrates will likewise show no toxic substance. The whole question of isolation of pure proteoses is much like that of the isolation of pure ferments. The process of isolation destroys the toxicity of the proteose and the activity of the ferment.

The "proteose solutions" employed in our experiments are prepared from the material obtained from human or animal intestinal obstruction or closed intestinal loops by means of alcoholic precipitation, solution of the precipitate in water, and removal of albumin by heat in a dilute acid solution (1). A second alcoholic precipitation may be used but this will destroy some of the original toxicity. The fluid is slightly opalescent or clear broth-like with a faint amber color. In the concentration used, the solution usually contains about 5 mg. of dried substance per cubic centimeter. We hope to report in the near future on a chemical study of this material. We realize that this preparation may contain one or more primary proteoses and perhaps some β -nucleoprotein and nucleohistone.

The important fact remains that the proteose solution contains a substance or substances which are not present in the normal intestine but are abundant in the obstructed intestine (human or animal). It is highly probable at least that this "proteose" is concerned in the intoxication of intestinal obstruction which is perhaps as typical an example as may be found of true non-specific intoxication. All infections have an important non-specific intoxication factor which may not be very unlike this non-specific intoxication of intestinal obstruction. We believe that information concerning the non-specific intoxication of intestinal obstruction will be of value for a proper comprehension of the non-specific fraction of the general intoxication present in bacterial infections.

Methods.

Urea was determined as described by Van Slyke and Cullen (4).

The amino nitrogen, peptide nitrogen, and total non-protein nitrogen were determined as follows:¹ 15 cc. of blood were treated for 15 to 30 minutes with 1 cc. of a 10 per cent solution of Squibb's urease. The proteins were then precipitated by diluting to 150 cc. with 2.5 per cent trichloroacetic acid. The filtrate was received in a measuring cylinder and the volume noted. When the drainage had practically stopped, the filtrate was transferred to a beaker and boiled 15 minutes to decompose the trichloroacetic acid. A few drops of saturated potassium carbonate solution were added to render the solution alkaline to phenolphthalein. The solution was then concentrated at 20–30 mm. pressure to remove ammonia and reduce the volume to a few cubic centimeters. It was finally transferred from the distilling flask to a 15 cc. measuring flask.

1 cc. duplicates were used for determination of total nitrogen by the micro-Kjeldahl technique of Folin and Farmer (5), the ammonia being titrated with 0.02 N acid and alkali. 2 cc. portions of the solution were used for amino nitrogen determination (6). For peptide nitrogen a 5 cc. portion was mixed with 5 cc. of concentrated hydrochloric acid and heated 24 hours at 100°C. to hydrolyze peptides. The greater part of the free hydrochloric acid was removed by concentration nearly to dryness under diminished pressure in a 50 cc. distilling flask. The residue was taken up with about 20 cc. of water, rendered alkaline to phenolphthalein with a few drops of saturated potassium carbonate solution, and concentrated again nearly to dryness to remove ammonia. The residue was brought to 5 cc. volume and 2 cc. portions were used for amino nitrogen determination, the value determined being that of the amino nitrogen plus the peptide nitrogen, which is converted into amino nitrogen by the acid hydrolysis.

EXPERIMENTAL OBSERVATIONS.

Dog 18–12 (Table I).—Mongrel, adult male. This dog was injected intravenously under ether anesthesia with a proteose solution prepared from material obtained from closed loops of the small intestine of the dog. A large dose, 260 cc., was given slowly and was associated with considerable fall in blood pressure. Vomiting and diarrhea appeared during the 1st hour after injection. During the 2nd hour the diarrhea became blood-tinged. Respiration was slow and deep; vomiting at intervals; pulse weak. After this the condition of the dog showed little change. The temperature showed slight rise with a fall shortly before death. 5.25 hours after injection animal was moribund; given ether and killed.

¹ The methods will be discussed in more detail in *The Journal of Biological Chemistry*. The present outline is, however, sufficient to permit repetition of the experiments.

Autopsy.—Typical of acute proteose intoxication described previously (7). Blood clots slowly. The spleen and liver are intensely engorged and deep purple in color. The duodenum, jejunum, and ileum show intense engorgement of the mucosa, which is velvety and deep purplish red. There is much fluid in the intestine.

TABLE I.

Dog 18-12. Acute Intoxication. Proteose Injection.

Time after injection.	Total non-protein nitrogen.	Non-protein nitrogen per 100 cc. of blood as.				Remarks.
		Urea.	Non-urea.	Amino nitrogen.	Amino nitrogen plus peptide nitrogen.	
hrs.	mg.	mg.	mg.	mg.	mg.	
Before.	45.5	10.8	34.7	11.0	18.8	Weight 26.3 lbs.
After.	47.9	11.5	36.4	12.2	22.4	Immediately after injection.
2.25	53.5	15.7	37.8	12.3	—	
3.75	62.6	20.7	41.9	13.2	25.8	
5.25	61.8	22.9	38.9	13.7	24.6	Autopsy typical.

Dog 18-19 (Table II).—Fox-terrier, adult female. Under ether anesthesia the proteose solution was given intravenously, 110 cc. in amount. The proteose was prepared from a human case of intestinal obstruction, material being removed at operation. Injection caused a moderate fall in blood pressure. Vomiting began within 30 minutes. After 1 hour a little semifluid feces was passed. The temperature became subnormal; pulse very weak. 5 hours after injection dog is prostrated; pulse barely palpable. 6 hours after injection dog in severe shock but would live perhaps 1 or 2 hours longer. Ether anesthesia; killed.

Autopsy.—Performed at once; picture identical with that described for Dog 18-12. Lesions are typical of an acute proteose intoxication.

Dog 18-25 (Table II).—Mongrel, young adult male. Under ether anesthesia a proteose solution, 175 cc., was injected intravenously. This had no effect upon the blood pressure. Vomiting began in 30 minutes and the temperature, which had risen slightly, fell gradually until death. The clinical picture is similar to that of the dogs described above. After 3.5 hours dog moribund and was killed.

Autopsy.—Performed at once; picture typical of proteose intoxication, as described for Dog 18-12.

Dog 18-28 (Table II).—Mongrel, young adult male. Under ether anesthesia a proteose solution, 150 cc., was injected intravenously. This proteose was obtained from a case of human intestinal obstruction. There was considerable fall of blood pressure during and after the injection. A progressive fall in temperature developed during the course of the intoxication. Vomiting began 20 minutes

after injection and diarrhea within an hour. The clinical picture is identical with that described above. After 4.1 hours the dog is in severe shock but might live 1 hour longer. Ether anesthesia; killed.

Autopsy.—Performed at once; findings are typical of acute proteose intoxication, as described for Dog 18-12.

TABLE II.
Acute Intoxication. Proteose Injection.

Time after injection.	Total non-protein nitrogen.	Non-protein nitrogen per 100 cc. of blood as.				Remarks.
		Urea.	Non-urea.	Amino nitrogen.	Amino nitrogen plus peptide nitrogen.	
Dog 18-19.						
hrs.	mg.	mg.	mg.	mg.	mg.	
Before.	33.0	9.0	24.0	9.9	16.4	Weight 13.4 lbs.
After.	37.1	10.1	27.0	10.8	18.7	
6.0	49.9	22.9	27.0	10.8	18.7	Killed.
Dog 18-25.						
Before.	—	—	—	—	—	Weight 22.5 lbs.
3.5	37.2	11.5	25.7	11.1	20.1	Killed.
Dog 18-28.						
Before.	—	—	—	—	—	Weight 17.3 lbs.
4.1	59.5	19.6	39.9	12.1	20.3	Killed.

Dog 18-21 (Table III).—Mongrel collie, adult female. Ether anesthesia. Laparotomy with section of small intestine 12 inches below the duodenojejunal junction. The sectioned ends turned in and united to produce complete obstruction. During the first 2 days after the operation the dog showed no clinical evidence of intoxication; no vomiting. On 4th day vomiting of bile-stained fluid began. There was slight fall in temperature. 5th day, vomiting continued. Dog is quite sick. 5 p.m. Ether anesthesia; killed.

Autopsy.—Performed at once. The peritoneal cavity contains 350 cc. of pale, thin, yellow, turbid fluid. Peritoneal surfaces show acute inflammation. A peritonitis resulted from necrosis of the intestine at site of obstruction with a slight escape of intestinal contents. The peritonitis is probably of short duration. Abdominal viscera negative except for cloudy swelling. Small intestine above obstruction shows definite engorgement of mucous membrane. There is little fluid in the intestine. Blood collected in dry oxalate shows little if any blood concentration.

Dog 18-50 (Table III).—Small bulldog, male; weight 12 pounds. Ether anesthesia, laparotomy, and production of complete obstruction in the middle of the small intestine. Obstruction produced by section of intestine and inversion of cut ends. Vomiting began on the 2nd day after operation. There is little intoxication until the 6th day; on the 7th day after operation dog is intoxicated, vomiting gray, foul smelling fluid. Pulse is weak. Ether anesthesia; killed.

Autopsy.—Performed at once. Wound is clean. Peritoneal surfaces smooth and moist. The small intestine above the obstruction is dilated and thickened.

TABLE III.
Intoxication of Intestinal Obstruction.

Time after operation.	Total non- protein nitro- gen.	Non-protein nitrogen per 100 cc. of blood as.				Remarks.
		Urea.	Non- urea.	Amino nitro- gen.	Amino nitrogen plus peptide nitro- gen.	
Dog 18-21.						
<i>days</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
2	—	7.9	—	—	—	Obstruction in midjejunum.
3	—	6.5	—	—	—	
5	—	55.2	—	—	—	10.30 a.m.
5	79.5	52.8	26.7	10.8	22.6	5.10 p.m. Killed.
Dog 18-50.						
7	137.2	97.9	39.3	9.6	17.0	Obstruction in low jejunum.
Dog 18-41.						
2	87.1	55.0	32.1	12.7	16.8	Obstruction in low jejunum.
Dog 17-222.						
25	39.2	16.3	22.9	4.3	7.9	Intestinal loop of ileum.

Other organs negative. The jejunum contained the usual amount of creamy-grayish, semifluid material which contains large amounts of toxic proteose.

Dog 18-41 (Table III).—Fox-terrier, adult male; weight 22 pounds. October 4, 1917. Ether anesthesia and extirpation of head of pancreas. Both arms of the pancreas isolated and left intact in the peritoneum. Dog recovered perfectly following this operation. November 6. Dog in good condition; weight 19 pounds. Ether anesthesia and simple obstruction in middle of small intestine. Day following operation animal is sick. Temperature normal. 48 hours after operation dog is severely shocked with subnormal temperature; would probably die within a few hours. Ether anesthesia; killed.

Autopsy.—Performed at once. Peritoneal cavity clear. The pancreas shows a good deal of induration and atrophy but the parenchyma is present in considerable amounts. The obstructed intestine contains about 450 cc. of creamy, brown, thick fluid.

Dog 17-222 (Table III).—Mongrel collie, female; weight 20.5 pounds. Ether anesthesia with isolation of a closed loop of the ileum beginning just above the ileocecal valve measuring 100 cm. in length. Ileum united around the loop by enteroenterostomy. Dog recovered well after operation. During the 2nd week occasional attacks of vomiting. There was evidence of chronic intoxication with gradual loss in weight. 25 days after operation weight was 17 pounds. Dog is not acutely intoxicated. Ether anesthesia; killed.

Autopsy.—Performed at once. Peritoneum is clean. The isolated loop contains 900 cc. of thick, creamy, brown fluid. The intestinal walls are hypertrophied and thickened. There is no ulceration of the mucous membrane. The rest of the gastrointestinal tract is negative. Other findings have no significance.

TABLE IV.
Normal Dog after Meat Feeding.

Time after meat feeding.	Total non- protein nitro- gen.	Non-protein nitrogen per 100 cc. of blood as.				Remarks.
		Urea.	Non- urea	Amino nitro- gen.	Amino nitrogen plus peptide nitro- gen.	
<i>hrs.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	Weight 14.1 kg. Fed 290 gm. of cooked ground beef heart.
0	29.7	9.8	19.9	10.1	15.8	
0.5	30.0	10.3	19.7	10.1	14.5	
1.5	38.3	15.3	23.0	10.7	15.5	
3.0	46.9	22.4	24.5	9.9	14.8	
5.0	51.7	28.4	23.3	9.6	15.1	
8.0	50.7	29.5	21.2	10.4	15.9	

DISCUSSION.

The results show that intoxication by injected proteose causes an immediate and rapid increase in autolysis of body protein. The effect is so marked that the increase in blood urea is comparable with that accompanying the digestion of a heavy feeding of meat. The other non-protein nitrogenous constituents are slightly increased, due almost entirely to increases in free amino acids (NH_2) and peptides (NH). The entire picture of the non-protein blood nitrogen is indistinguishable from that following a heavy protein meal (Table IV).

The same remarks apply to the intoxication following intestinal obstruction. The urea is enormously increased over the usual fasting value, indicating a protein catabolism so rapid that the kidneys fail to keep pace with it. The non-protein nitrogenous products of the blood other than urea are not appreciably altered.

There is no evidence to indicate that the intoxication results from the tissue autolysis. The reverse appears to be the case, because in no instance does the urea concentration reach a toxic level, while the other nitrogenous products are not increased at all beyond usual limits. Also, in Dog 18-25, Table II, death occurred before autolysis had gone far enough to raise even the urea beyond that observed in fasting.

On the other hand, the results present a good example of accelerated protein catabolism and tissue autolysis caused by the action of a toxin, uncomplicated by the presence of parasites, by abnormally high temperature, or by any other apparent factors save the toxin itself.

The fact that the peptide nitrogen of the blood is not increased to an abnormal degree by the intoxication does not exclude the possibility that among the products of the induced autolysis there may be toxic proteoses which add their effect to that of the injected or absorbed proteose. The amounts of such proteoses required to intoxicate are too little to increase measurably the peptide nitrogen of the blood.

SUMMARY.

The acute intoxication following an injection of a toxic proteose is usually associated with a large increase (40 per cent or more) in the non-protein nitrogen of the blood. This increase is found chiefly in the blood urea nitrogen, but the amino and peptide nitrogens also may show small increases. The changes observed in the blood non-protein nitrogen are identical with those which follow the feeding of large amounts of meat (8).

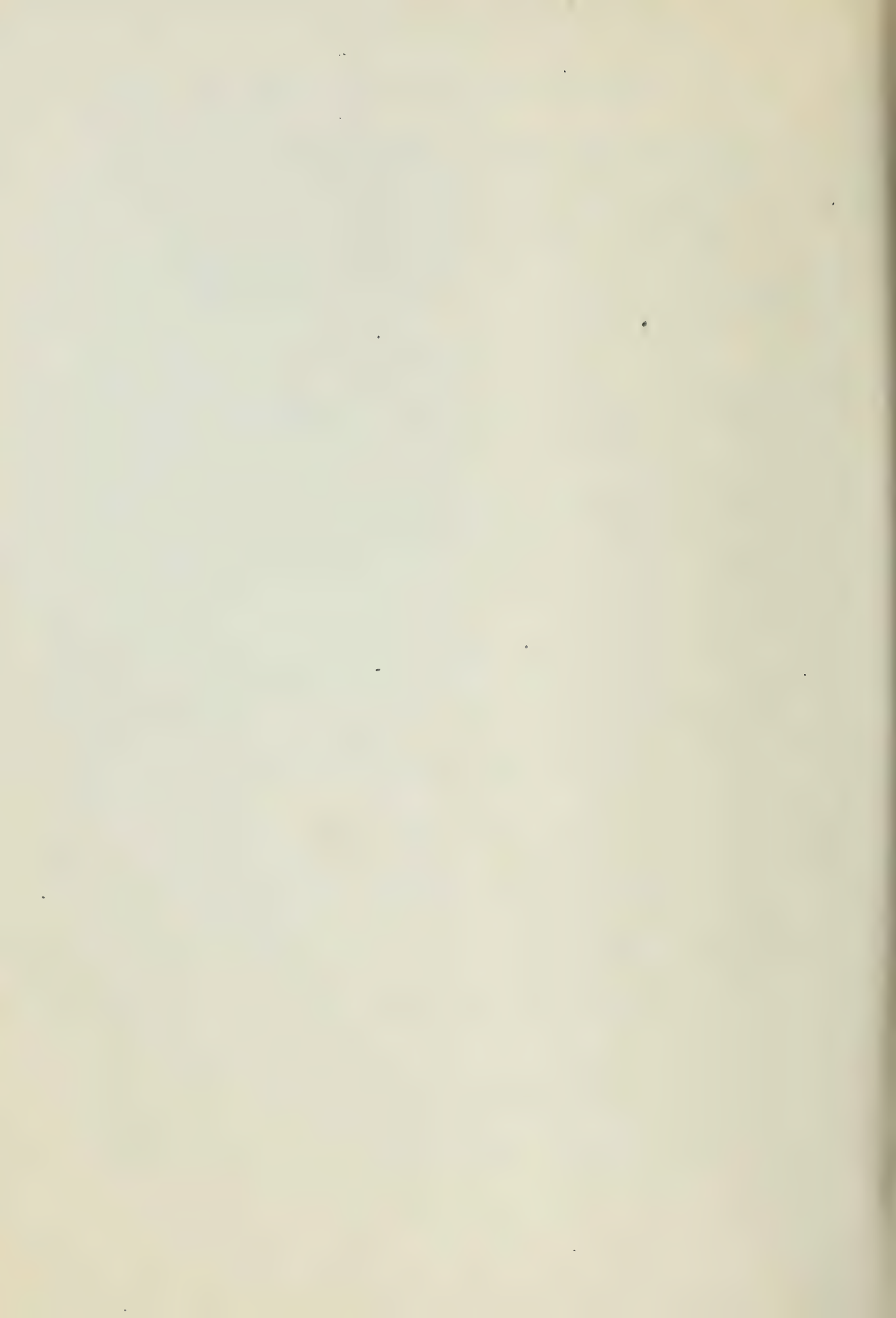
These facts indicate that the proteose intoxication causes an abnormally rapid autodigestion of tissue proteins, but that the nitrogenous end-products are, in chief part at least, the same that result from normal catabolism of food proteins. There is no evidence that the

autolytic products play any part in causing the intoxication. The possibility of such a part and a resultant vicious circle is not excluded, but from the available facts the autolysis appears more as a result rather than cause of the intoxication.

It appears possible that in disease or intoxication tissue catabolism may be enormously accelerated and yet yield the end-products of normal protein metabolism.

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TREATMENT OF EMPYEMA BY THE CARREL-DAKIN
METHOD AT THE WAR DEMONSTRATION HOS-
PITAL, THE ROCKEFELLER INSTITUTE FOR
MEDICAL RESEARCH.*

PRELIMINARY REPORT.

By GEORGE A. STEWART, M.D.

Major M. R. C., U. S. Army.

In the War Demonstration Hospital at The Rockefeller Institute for Medical Research we have treated a number of cases of empyema following pneumonia. As many of these cases have been of a peculiar type an entirely new problem has been presented. All have been treated with the Carrel-Dakin method. These cases had first been admitted to the Rockefeller Hospital as pneumonia cases and then transferred to the War Demonstration Hospital after the development of empyema.

As this is to be a discussion on treatment, I will merely mention that the usual physical examinations, x-ray, thoracic punctures, and cultures of the pleural fluid, were employed in all cases for diagnostic purposes. The case was considered one of empyema if on thoracic puncture macroscopic pus was found, or if pus cells and organisms were demonstrated by the microscope.

This evidence was considered sufficient for operation, and some cases were operated on as early as the third day after the onset of the pneumonia. The longest period which elapsed before operation was 120 days. This patient had been aspirated in another hospital seven times. Our operations were performed under local anesthesia, novocaine 1:400. The operation of choice has been rib resection, opening into the pleural cavity and exploration with the gloved finger or instrument, opening all pockets. The site of operation in those cases where the fluid seemed to be evenly distributed has been

* Read at the meeting of the American Association for Thoracic Surgery, June 10, 1918.

at the lowest point posteriorly, and in most cases over the eighth or ninth rib in the posterior axillary or scapular line. In those cases where a definite localized pocket was found the opening was made over the region of the pus.

In the early cases the pleural cavity was not flushed out at operation. Three to five Carrel perforated tubes were placed in the cavity extending to all points, and two large short tubes inserted in order to have a free outflow of secretion and chlorinated material. With our later cases the pleural cavity has been flushed with Dakin solution at the time of operation, the large tubes have been omitted and four Carrel tubes stiffened with fine silver wire have been placed, endeavoring to reach all parts of the cavity. Compresses wet with Dakin solution, replaced the large tubes originally used, in order to prevent a too rapid interchange of air. After placing the tubes and compresses, x-ray examination showed that the tubes reached all parts of the cavity.

In the early cases instillations of 30 to 60 cc. of 0.2 per cent. sodium hypochlorite solution were given every two hours for forty-eight hours, and as no contraindications arose in the use of this solution the strength was increased to 0.5 per cent. sodium hypochlorite, or full-strength Dakin solution. In our later cases instillations have been given every hour during the day and every two hours during the night, and the amount has been increased to from 80 to 100 c.c. Dakin solution. This has been done to follow more closely the necessary principles of time, concentration, and contact in the use of an antiseptic.

Dressings are made daily. A smear from the inside of the pleural cavity is taken to be examined microscopically, the cavity is flushed out, the individual tubes are tested, and fresh tubes inserted when necessary. Compresses wet with Dakin solution are then placed in the external wound as at operation, the skin is protected with vaseline compresses or zinc oxide ointment, and a cotton pad and chest binder applied. The external dressings are changed by the nurse when necessary.

In our first cases closure took place spontaneously in from three to seven weeks, no attempt being made to suture the wounds. In the later cases, however, we have made secondary closure with suture

when sterilization was obtained as shown by the bacteriological curve, and in some cases by culture as well. Cultures taken at the time of dressing have been found sterile at the end of five to nine days. In this way we were able to close cases in from five to twelve days or more, the average time being about fourteen days. Primary union was obtained in about 70 per cent. of the cases. When secondary sterilization was necessary this was readily obtained in all cases in a week or less, and complete closure was the final result. The secondary infections were always found to be of a low virulence.

After operation the patients were comfortable and were, in general, free from the toxic symptoms present in most empyema cases. The secretions have been small in amount and free from odor. The temperature has dropped, as a rule, after operation, and in a few cases where it has remained elevated this has been due to the pneumonic process which was still active, or else to complications, since practically all of our cases were operated upon before a crisis or lysis.

We have treated up to the present time forty-five cases. With the exception of five of these cases the entire treatment was carried out in our hospital. These five cases were sent to us from other hospitals with sinuses that had been discharging from three days to three months. The ages of the patients have varied from fifteen to sixty-eight years. We have had thirty-two cases of streptococcus infection, nine cases of pneumococcus infection, and four cases in which it was impossible to isolate any one type of organism.

At operation we have found in the great majority of cases the fluid to be perfectly free in the pleural cavity. The character of the fluid was usually thin and watery, of a greenish, yellowish, or brownish color, and in some few cases thick, creamy pus. In a number of cases a large amount of fibrin was present, covering the pleural surfaces, and with irrigation large shreds were washed away.

Complications other than those to be mentioned in autopsy findings have been three cases of abscess of the buttocks, three cases of acute tonsilitis, and one case of scarlet fever. We have had twelve deaths in this series, nine of which have been of the streptococcus groups. At autopsy we have found pericarditis in five cases, together with one or more of the following lesions: cervical adenitis, mediastinitis, miliary tuberculosis, intestinal ulcerations, peritonitis,

numerous small lung abscesses, and small kidney abscesses; one case of general tuberculosis, three cases with pneumonia involving the opposite lung, and a beginning fibrinous pleurisy. In three cases we were unable to obtain autopsies and cannot state the exact condition present.

No case has been discharged from the hospital with a sinus. All wounds have healed clean and solid. Chest examination has shown the lung to be practically normal, judging from physical and x-ray examinations. We have been able to return cases to duty in twenty-one days after operation. One case had pneumonia, empyema, operation, sterilization, closure, and was ready for duty in twenty-six days. The average length of time in the hospital after operation has been fifty days. This seems rather long, but we have often kept cases several weeks for demonstrative purposes. Not a case, to our knowledge, has been discharged from the Army or Navy for physical disability. All have gained in weight—one as high as 36 pounds. We have discharged in all twenty-two cases and some of our soldiers are now in France.

In this small series of cases, summarized in a brief preliminary report, we have found it possible to use the Carrel-Dakin method of treatment of empyemas with very satisfactory results when careful attention has been paid to all the details of its application. The series of cases has not been large, but the ultimate results obtained—forty-five cases treated, twelve deaths, eleven still in the hospital under treatment (five of which are about ready for discharge), and twenty-two cases discharged, the comparatively short stay of patients in the hospital, and their ability to return to active duty—well demonstrate the value of the method in the treatment of empyema cases.

COCCIDIOSIS IN YOUNG CALVES.

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PLATES 1 TO 3.

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Among protozoan parasites coccidia have received considerable attention because the asexual and sexual cycles are readily available for study. They have served to demonstrate the remarkably complex life cycle of certain groups of protozoa. They also possess a certain practical importance since they may produce diseases, at times of epizootic proportions, among domestic animals. Coccidiosis as a disease in cattle was first brought forward in 1892 by Zschokke¹ and Hess² who observed it in Switzerland. The name *rothe Ruhr* was suggested by the discharges of blood in the feces. Before this Zürn³ in 1878 had reported coccidia in the intestines and mesenteric glands of a calf which died as a result of a severe enteritis. Since 1892 coccidia have been found in cattle by various observers, especially in tropical and subtropical countries and to a slight extent in temperate climates (Denmark, Russia, France, Italy, and Germany). So far as it is possible to consult the original literature, no studies of the cases found were made beyond the microscopic examination of the feces except by Züblin⁴ who undertook a fresh investigation of certain cases of *rothe Ruhr* in Switzerland in 1906 and whose work is referred to farther on.

In the United States up to 1911 the presence of coccidiosis had not been noted. In a general article on the subject Crawley⁵ writes as follows:

¹ Zschokke, E., Beobachtungen über die rothe Ruhr, *Schweiz. Arch. Tierheilk.*, 1892, xxxiv, 1, 49.

² Hess, E., Die rothe Ruhr des Rindes. (Dysenteria hæmorrhagica coccidiosa.), *Schweiz. Arch. Tierheilk.*, 1892, xxxiv, 105.

³ Zürn, F. A., Vorträge für Thierärzte, 1878, i, No. 2.

⁴ Züblin, E., Beitrag zur Kenntnis der roten Ruhr des Rindes (Dysenteria coccidiosa bovis), Inaugural dissertation, Zurich, 1908.

⁵ Crawley, H., *Twenty-Seventh Ann. Rep. of Bureau of Animal Industry, U. S. Dept. Agric.*, 1910, 488.

"*Coccidium zürni*.—This coccidian is considered to be the cause of red diarrhea of cattle. The disease has been noted in Europe, where outbreaks of a similar character have also occurred among sheep. The lethality, however, for cattle seems to be rather low, varying from 2 to 4 per cent. As it is the intestinal cells which are attacked by the parasite, the mucosa of the intestine becomes stripped off in places, resulting in extensive hemorrhage into the lumen, which causes the red diarrhea. In fatal cases, death results within 2 days. Most of the recorded cases have occurred in Switzerland, in summer and autumn. Dampness favors the disease, probably for the reason that in dry weather the encysted infective stages of the parasite become desiccated, and thus many are killed."

In September, 1915, Schultz⁶ stated that coccidiosis existed among cattle in the Northwest of the United States without, however, supplying any detailed data.

Dr. Schultz kindly sent some slides of what appeared to be washed feces to us recently. A careful examination by two observers independently resulted in the finding of a few undoubted oocysts. These would demonstrate the existence of the parasite in the Northwest. In other slides in which schizonts and merozoites were supposed to be present only desquamated epithelium was found. The character and intensity of the western disease needs, therefore, further elucidation.

In the prosecution of certain researches upon the diseases of calves in New Jersey, coccidiosis was encountered unexpectedly in association with other infectious diseases. Though the material available for study was not extensive, it became evident that coccidiosis might by itself prove fatal to calves, and the subject was deemed of sufficient importance, therefore, to be discussed independently of other associated calf diseases.

General Characters of the Disease.

Epidemiology.—The first case containing coccidia was autopsied April 14, 1917. Other cases followed during the spring and summer. A few coccidia were found in a calf as late as December 2. The severest, fatal cases occurred in summer. The invasion probably begins soon after birth and continues over a period of 6 to 8 weeks at the

⁶ Schultz, C. H., Observations among dairy cattle, read at the Annual Convention of the Washington State Dairymen's Association, Snohomish, December 15-17, 1915.

longest, unless death occurs in the meantime from this or other affections. Blood in the stools, being the characteristic sign which probably appears when the first crop of oocysts is being discharged, occurred anywhere from 3 to 6 weeks after birth. The fact that the affected calves were not under our immediate care makes any statement bearing on the clinical course subject to corrections. In several severe cases blood in the feces was not reported.

Evidence of active multiplication of coccidia in cattle older than 3 months is not at hand. The disease appears to exert itself chiefly upon the very young, the older animals having acquired a certain resistance.

Infectiousness does not seem to be very great. The calves were kept together in lots of six or less in small indoor enclosures and were allowed to run together in small yards. Assuming that the oocysts passed in the feces become infectious within a few days in warm weather, there was a striking difference in the susceptibility of different calves of the same lot. The presence of pneumonia or interstitial nephritis did not seem to affect the susceptibility in one direction or the other.

Symptoms.—The clinical examination of the calves was not carried out in any systematic way because they were not at any time during life on the Institute grounds. It will be noticed by consulting the protocols that certain animals were affected with pneumonia and with a sclerosis of the kidney which complicated a study of those manifestations due directly and exclusively to the coccidiosis. The discharge of blood in the feces was the only characteristic sign. In nearly all these cases the oocysts were found in the masses of blood (Fig. 1). The few observations made during the life of the calves are noted under each case.

Inasmuch as Züblin had under observation cases at first uncomplicated with other infections, his clinical notes are of value to us. He does not mention young calves as subject to this disease. His cases were chiefly pastured animals and in one herd of ten head of cattle, all of which were affected, the ages ranged from $\frac{1}{2}$ to 2 years.

According to Züblin, the first sign of disease is the passage of blood clots, about 10 to 15 cc., in normal feces by clinically normal animals. 1 or 2 days later blood is more abundant, defecation more frequent. The case may now return to normal

or it may grow worse. Usually the older animals recover promptly. In younger animals the quantity of blood passed may increase up to a man's fist in size. The tenesmus becomes more intense and the feces tend to become thin, foul smelling, the temperature rises, respirations are increased in number, and the appetite is abnormal. From the 5th to the 8th day the admixture of blood in the feces is replaced by mucus and masses suggesting croupous inflammation of the large intestine. The feces are watery and putrefactive. All other symptoms are accentuated and fever is present. With the loss of blood and refusal of food the animal becomes very weak and death occurs often as early as the 7th day. The mortality in Züblin's cases was 5 per cent.

The symptoms in his cases point to septic infection as the probable cause of death. It is obvious that the mortality rate in this disease will depend largely upon the presence of septic organisms in the herd and their relative degree of virulence, for the coccidia themselves do not cause inflammatory reactions but simply open the way for bacterial infection.

Diagnosis.—The feces were examined directly when blood or mucus or both were accessible, by placing a particle of the suspected material on a slide with or without a drop of salt solution and examining under a cover-glass with low and high powers. The oocysts are sufficiently characteristic to be identified under high dry objectives, and they are often recognizable under a low power. Drying and staining should not be resorted to.

When the feces are free from blood and coccidia are not detected directly, the following method for concentrating the feces which is a simplified modification of Hall's method⁷ and which was used in our examinations will be found useful:

A mass of feces is thoroughly shaken in a closed Mason jar partly filled with water, until well broken up. Then the jar is completely filled with water and the feces are permitted to settle. The water is poured off and the jar again filled with water. The sedimentation, decantation, and washing are continued until the water is nearly clear. In this way the particles lighter than water are removed. The sedimented feces are next mixed in a jar with about enough water to fill a cylinder in which they are to be sedimented and are poured through a tea-strainer held over the cylinder. By stirring the portion of the feces held back in the strainer and by dipping the bottom of the strainer up and down in the water after it nearly reaches the top of the cylinder, oocysts that have been held back are permitted to pass through. After the feces have settled to the bottom, a sample

⁷ Hall, M. C., A comparative study of methods of examining feces for evidences of parasitism, *U. S. Dept. Agric., Bureau of Animal Industry, Bull. 135*, 1912.

is taken with a pipette of wide caliber, mounted, and examined. By this method a few oocysts were brought to light in normal stools from calves apparently in good health.

After death the gross appearance of the large intestine does not offer any characteristic features for the recognition of the invasion. Two indications might be looked for: a change in the appearance of the mucosa due to the mechanical presence of large numbers of coccidia, and changes due to pathological alterations of the invaded tissue. As regards the first clue nothing is deducible from our observations. It may be stated here that in other species indications may be present. In a case of coccidiosis of the small intestine of a sheep seen by one of us many years ago, the foci were readily detected by the unaided eye. They were circular, opaque, whitish, slightly elevated patches up to a centimeter in diameter contrasting sharply with the normal pinkish mucosa. The slight thickening of the patch was due to the fact that every epithelial cell of the villi contained several coccidia, and as a result the affected villi became giants as compared with the normal. It should be stated that this animal had been killed and postmortem changes were absent. With regard to the second indication, the presence of one or more gross pathological changes, it may be said that the severest of the cases observed died in hot weather and came to autopsy when postmortem changes were well under way. Congestions and scattering punctiform hemorrhages into the mucosa were the most definite alterations noticed. The diagnosis will in all cases depend on the microscopic examination of the feces, or on sections of the large intestine, or on both combined.

The Parasite.

The Oocysts in the Feces.—In the course of the work it became evident that there were two species of coccidia infesting the calves. The species first encountered and present in large numbers is distinguished by a small oocyst usually elliptical but occasionally ovoid or circular in outline. The wall is double contoured, uniform in thickness, and as a rule thinner than the wall of the oocyst of the other species. Oocysts were encountered in which the protoplasm completely filled the cyst wall. All stages to that in which the protoplasm

was contracted to a circular mass removed from the cyst wall or touching it only at one side have been encountered. When the protoplasmic mass is well contracted it is located as a rule near the center of the cyst, yet it may be in contact with one side of the wall or nearer one end than the other. The cytoplasm contains highly refractive granules varying in size which may be uniformly or irregularly distributed through it. The nucleus is sometimes visible. Fifteen oocysts were measured from Calf E. The length ranged from 14.6 to 20.5 microns, and the width from 12.3 to 16.4 microns. Eleven oocysts were measured from Calf 102. The length ranged from 13.1 to 28.7 microns, the width from 12.3 to 20.5 microns. Ten cysts were measured from Calf 121. The length ranged from 16.4 to 20.5 microns, the width from 13.1 to 16.4 microns. The average length of the above thirty-six oocysts was 18.6 microns, the average width 14.8 microns.

The second species characterized by large ovoid oocysts was first encountered in Calf 92, dying June 6, and thereafter in most cases, either in the feces, or in fresh scrapings of the mucosa, or in sections. The oocyst is distinctly ovoid in shape. Sometimes the cyst wall is brownish in color, at other times it is colorless. The wall is thickest at the broad pole and very gradually diminishes in thickness to the small pole. The difference at the two poles is not great yet distinctly noticeable. Some cysts occur, however, in which the wall is uniform in thickness. There is no evidence of a micropyle.

The cytoplasm as a rule is contracted into a circular mass located at the middle or in the broader portion of the cyst (Fig. 6). It occupies two-thirds to three-fourths of the space within the wall and comes almost in contact with the sides. The cytoplasm is filled with large highly refractive granules. The nucleus is not visible. Thirteen oocysts were measured from Calf B. The length ranged from 25.8 to 41.8 microns and the greatest width from 17.2 to 24.6 microns. Fifteen cysts were measured from Calf E. The length ranged from 28.7 to 32.8 microns, the width from 16.4 to 20.5 microns. The average length of the above twenty-eight oocysts was 29.9 microns and the average width 19.9 microns.

Development of the Oocysts in Cultures.—In order to determine certain points in the development of the oocysts, feces, blood, and mucus

containing them were streaked over the surface of agar plates.⁸ The surface of the plates was kept moist by adding from time to time a little distilled water. They were kept in a moist chamber at room temperature.

In the large, ovoid species the mother sporoblast or oocyst produces four spores without leaving behind a so called *Restkörper*. The spores are elongated, nearly elliptical in outline, circular in cross-section, and taper a little toward one end. The spore is surrounded by a thin, single contoured membrane which is thickened to form a cap-shaped body at the smaller pole. There is a large *Restkörper* within the spore. It is elliptical or fusiform in shape and lies against the wall of the spore, sometimes in a space formed between the sporozoites, in which case it occupies a diagonal position with respect to the spore. The *Restkörper* contains rather large, round, highly refractive granules of approximately the same size.

There are two sporozoites in each spore and these with the *Restkörper* completely occupy the space within the limiting membrane. They are broad and rounded at one end and taper toward the other end. The broad ends of the sporozoites are at opposite ends of the spore and the contact between the two is marked by a line running diagonally across the spore. The position of the nucleus varies somewhat. It is either located nearer or in the broad end of the sporozoite. It appears as a small halo, enclosing a minute, round nucleolus. The cytoplasm is minutely granular and is uniform in appearance throughout the sporozoite. There is little variation in the size of the spores. Eight spores were measured in the cysts. The length ranged from 16.5 to 17.1 microns, and the width from 7.2 to 7.6 microns.

In the small species with ellipsoidal oocyst four spherical sporoblasts form which become elliptical in outline and develop into the spores (Figs. 2 and 3). These are elongated, elliptical, and, as in the large species, they taper a little at one end. The spore is circular in cross-section. It is surrounded by a thin, single contoured membrane which is thickened into a minute cap at the small pole. There is no *Restkörper* left behind in the formation of the sporoblasts. There is no

⁸ This medium consisted of 20 gm. of agar-agar, 5 gm. of sodium chloride, and 1,000 cc. of distilled water. The agar-agar is cut up, tied in a gauze bag, and washed for 2 hours in running water before the medium is made up.

Restkörper in the spore. The sporozoites are the same shape as in the large species. They are round at the broad end and taper toward the other. In one instance the tapering end was seen to be pointed. In the middle of the sporozoites or possibly more frequently in the large end is generally found a rather large, more or less well defined mass of very minute granules. Elsewhere the protoplasm is homogeneous in appearance. The nucleus is located nearer the broad end and consists of a small, round granule surrounded by a halo. The line separating the sporozoite runs diagonally across the spore. The sporozoites completely fill the spore.

The spores do not vary much in size. They are much smaller than those of the large species. Seven spores were measured. The length ranged from 9.9 to 11 microns, and the width from 5.3 to 5.7 microns. The results of the various measurements are brought together in Table I.

TABLE I.

Species.	Oocyst.		Spore.	
	Average length.	Average width.	Length.	Width.
Ovoid.	29.9 microns.	19.9 microns.	16.5-17.1 microns.	7.2-7.6 microns.
Ellipsoidal.	18.6 "	14.8 "	9.9-11 "	5.3-5.7 "

Studies to determine the exact time required at certain favorable temperatures from the discharge of the oocyst to the maturation of the sporozoites have not yet been made. Approximate estimates based on cultures and subject to revision indicate that in midsummer this portion of the cycle of the ovoid type covers about 5 days. Similar estimates indicate a somewhat longer period for the ellipsoidal type.

Von Wasielewski⁹ states that the oocysts of *Eimeria cuniculi* vary in size and form within wide limits. Cysts occurred the volume of which was only a fraction of the average size. The ripening of these small cysts was normal. Similar variations in size were encountered by him among the oocysts of *Diplospora bigemina* from the cat's intestine. Züblin likewise refers to variations in size corresponding fairly well with our types. Thus in rare cases, he states, the width was 20 microns,

⁹ von Wasielewski, T., Studien und Microphotogramme zur Kenntnis der pathogenen Protozoen, Leipsic, 1904, 1. Heft.

the length 30 to 35 microns. The largest number were roundish with a mean diameter of 12 to 15 microns. Nothing is stated of morphological differences between these extremes. It may therefore be claimed that the inference that there were two species of coccidia in the calves is not called for. Pending further investigations it may be pointed out that forms intermediate in both shape and size between the two types of oocyst were not encountered. The small ellipsoidal form was the numerically predominating type and in a few cases the one exclusively seen. The presence of a *Restkörper* in the large, and its absence in the small oocyst is noteworthy. Seeing these two forms in the same field of the microscope, the observer is led to the conclusion that the burden of proof that they are variants of the same species must rest with the one who supports such a view. Anticipating somewhat, we may state that careful study of the sections did not enable us to distinguish between these species in the schizogonic cycle. This may have been due to the relative scarcity of the larger parasite in the material at hand. In any case the final decision will require a study of fresh material on a relatively larger scale.

Among those who have reported on the presence of coccidia in cattle, Guillebeau,¹⁰ Züblin, and Jowett¹¹ have attempted cultures of the oocysts. Züblin describes the development of the four spores and eight sporozoites as we have found it in the ellipsoidal form. But in addition he describes forms as going through the stages of schizogony in his cultures—forms which may have been other protozoa, such as amebæ, or fungi and of which we also have found multiplying forms in cultures. Further material will be needed to determine whether Züblin actually found a hitherto undescribed stage in fecal cultures or whether he simply described stages of coexisting organisms. Jowett¹¹ (South Africa) describes a case of bloody dysentery in a calf 3 months of age. The coccidia were round or subspherical. Distinctly oval cysts were rare. The smallest measured 14.4 by 12.8 microns, the largest, 27.2 by 20.8 microns. No details are given except that four sporocysts form, within which sporozoites appear.

The Parasite in the Mucous Membrane.—Owing to the fact that the material on hand coming from animals killed was meager and that the heaviest invasion occurred in animals which died and in which postmortem changes were under way, any detailed description of the stages of the cycle within the host will be postponed. The barest outline must suffice here.

It may be said, however, in spite of the deficiency of material that nothing has been seen which suggests that the coccidia encountered depart in any significant way from the cycle as mapped out for such a well known coccidium as that of the rabbit.

¹⁰ Guillebeau, cited by Züblin.⁴

¹¹ Jowett, W., Coccidiosis of the fowl and calf, *J. Comp. Path. and Therap.*, 1911, xxiv, 207.

It is believed that the stages that have been encountered and described belong to the small elliptical species, for the following reasons: first, the small species, as shown by the number of oocysts in the sections, occurs in very large numbers whereas the large species is relatively rare; and second, no anomalies have been encountered leading one to believe that stages of two forms so different in size were being studied.

The earliest stage observed in epithelial cells is roundish or elliptical in outline. The nucleus is centrally or somewhat eccentrically situated and consists of a well staining karyosome surrounded by a halo usually ill defined. However, a halo is not present with all nuclei. The cytoplasm stains lightly and is alveolar in structure. The roundish forms measure about 6 microns and the elliptical ones 5 to 6 microns long and 4 to 5 microns broad.

Larger uninuclear forms were encountered which the evidence favored interpreting as schizonts. They are elliptical in shape or often compressed laterally in the epithelial cell, then presenting an oblong appearance with the sides more or less straight. The cytoplasm shows the same, or approximately the same structure as that in the multinuclear schizonts and there are no metachromatic granules present. These occurred in crypts where schizogony was general and where there was no definite evidence of sporogony. They measure 8 to 10 microns long and 6 to 7 microns broad.

Forms containing two nuclei are circular or elliptical in outline, there is no cell membrane visible, the cytoplasm is alveolar, and the nuclei consist of a karyosome which may or may not be surrounded by a halo. The elliptical forms measure 5 to 7 microns long by 4 to 6 microns broad.

Forms containing from three to seven, nine, and eleven nuclei have been observed but no attempt has been made to determine the number of nuclei in more advanced forms than these. They are circular or elliptical in form and devoid of a cell membrane. Some schizonts stain more deeply than others and the cytoplasm appears more compact, being almost homogeneous. However, as a rule the cytoplasm stains lightly and is alveolar in structure. The daughter nuclei are located at or toward the periphery of the parasite. Typically the nuclei show a dark staining karyosome surrounded by a rather ill defined halo. In some instances a halo about the karyosome cannot be distinguished.

The next stages seen are schizonts in which the cytoplasm has divided to form the merozoites. The merozoites, as shown in Fig. 5, are elongated, banana-shaped bodies usually lying with long axes nearly parallel and enclosed in a vacuole of the host cell. The nucleus is located at the middle. The entire mass representing the schizont measures 9 to 11 microns. The number of merozoites varies; five intact groups contained 13, 18, 18, 18, and 26 merozoites.

The gametes are usually found among schizonts in a later stage of the parasitism. Macrogametes, microgametocytes, and oocysts may be found in the same tubule. The earliest stages of the gametes it has been impossible to distinguish clearly from young uninuclear schizonts. The cytoplasm of forms believed to be

macrogametes, however, is more compact and stains more deeply than that of the schizont. Soon the enlarging body with nucleus remaining single, the circular or plump oval form, and the presence of metachromatic granules give a specific character to the macrogametes. There is no cell membrane. The nucleus as a rule is eccentrically located. It consists of a karyosome surrounded by a halo which in some instances is very large having a diameter of more than half that of the cell. Stages from 4 to 10 microns in diameter have been studied. Forms 6 microns in diameter have been seen containing metachromatic granules, although there seems to be some variation in the time at which these granules appear, larger forms having been seen which showed none or only very few. The granules are round and vary greatly in size not only in different individuals but also in the same one. They have also exceptionally been found persisting in the oocysts after the cytoplasm has retracted from the enclosing wall. In the largest macrogametes a cell membrane is present and the nucleus may be indistinct.

At a fairly early stage the nucleus of the microgametocyte has divided and many coccus-like bodies which stain intensely in basic dyes are found arranged uniformly or irregularly at the periphery of the cytoplasm. These nuclei of the future microgametes gradually change into very fine, hair-like, slightly wavy bodies which project on all sides from the cytoplasm. Well advanced microgametocytes are circular or elliptical in shape. The cytoplasm is more or less vacuolated and stains irregularly. Those of a circular shape measure 7 to 9 microns.

The development of the shell of the oocyst takes place gradually. The oocyst is first surrounded by a very thin membrane. Later a somewhat thicker single contoured membrane is present which stains with acid stains and increases in thickness until the fully developed, highly refractive, double contoured cyst wall is formed. When the enclosed cytoplasm has retracted from the cyst wall it is seen to be still surrounded by a delicate membrane. Various conditions of the oocysts are found in the epithelial cells, in the lumen of tubules, or in the midst of dislodged epithelial cells. The wall is often crumpled. The cytoplasm may completely fill the space within the cyst wall, may be slightly retracted from it, or may be contracted to a spherical mass occupying approximately a central position. The cytoplasm is most often dense and presents a minutely granular appearance, but in some instances it is alveolar in structure. Metachromatic granules are sometimes present in the peripheral portion of the cytoplasm. The nucleus as a rule stains well and uniformly.

The parasite acts on the host cell by absorbing its nutrient fluids and encroaching on the cytoplasm. There is, however, no change in the cytoplasm immediately surrounding the parasite, leading one to believe that the parasite does not exert a digestive or coagulative influence on it. In rare instances the parasite is surrounded entirely or in part by a clear space, but this has come about through a shrinking of the parasite. This condition is rare in material studied from calves that had been killed and the tissues fixed at once, but in material from calves

that died, in which the tissues were subjected to postmortem changes, it is common, and the smaller size of the schizonts and the compact condition of their protoplasm indicate that it has been brought about by shrinkage.

The nucleus of the host cell may not show any changes at first. Later its affinity for stains may be reduced or almost completely destroyed, and the chromatic masses may be reduced in number or disappear entirely. When a parasite lies contiguous to the nucleus, even though it is an early stage occupying only a small fraction of the cell, the nucleus is compressed and may even present a crescentic form.

Pathology.—The seat of the disease in the calves was the large intestine. In the small intestine scattering tubules were found invaded by one or a few parasites, rarely by many, even when the large intestine was severely involved. The forms encountered were oftener gametes than schizonts. In some tubules both sexual and asexual stages were intermingled.

In the large intestine the infection appeared at first in circumscribed foci. These foci tend to grow larger and in some cases coalesce with one another. Usually a given stage, such as schizogony, prevailed in a focus. Later both sexual and asexual forms were found together. In the same tubule may then be found fully developed merozoites, macrogametes, microgametocytes, and oocysts. The fertilized oocysts are probably discharged very promptly, for they are not abundant in the tubules. While schizogony is going on, oocysts may be discharged in large numbers in the feces. This was seen in Calf 121.

Not infrequently there may be seen between tubules thoroughly infected with schizogonic forms, scattering empty shells of oocysts which are embedded in the intertubular tissue. They are not carried there by manipulations, such as section-cutting, since some of them are within phagocytic cells. These shells represent aberrant parasites which have migrated through the epithelium, have gone through the various stages, and cannot be discharged outward. These aberrant cysts are relatively rare but wherever they occur in association with active schizogony in the tubular epithelium they prove that the disease started in an earlier invasion and that the present schizogony is either the result of fresh infection or else it represents a schizogony continuing from the beginning of invasion.

The topography of the invasion and the stages found in a focus give us a tentative explanation of the way in which the infection spreads. A sporozoite entering a tubule of the large intestine produces about sixteen merozoites which are discharged into the tubule and which infect the neighboring cells. These produce each sixteen merozoites which spread to neighboring tubules centrifugally. If the invasion is heavy and a large number of foci started, these may coalesce and form large patches. When the multiplication has gone on for a time the cycles intermingle and various stages, as stated above, will be found in the same focus.

The relative immunity of the small intestine as compared with the large intestine merits attention. In rabbits and birds (sparrows, hens, turkeys, and pheasants) the principal seat of invasion is the small intestine, and only secondarily the large intestine and ceca. Evidently the conditions in the small intestine are unfavorable in calves. Whether the difficulty is mechanical or physiological, that is, due to the active movements of the tube or to the digestive ferments, remains to be determined.

A more interesting problem in immunity is the self-limitation of the disease in a number of cases and the apparently complete immunity of some calves penned with the severe cases. On page 106 are given brief notes of certain calves which had bloody evacuations associated with large masses of oocysts and which recovered completely. Other calves associated with them remained unaffected and samples of feces concentrated before examination by washing and sedimentation did not reveal any coccidia. It is conceivable that schizogony is a self-limited process. If this is true, immunity would play no important part and the extent of the disease would depend upon the size of the infecting dose. Inasmuch as acquired immunity does play a certain part in the diseases due to protozoa living free in the blood and in blood cells, it cannot be at present ignored for coccidiosis. For practical purposes the infecting dose should be kept as small as possible, if it cannot be entirely eliminated, until its relation to the severity of the resulting disease has been more definitely formulated.

The injuries to the host caused by coccidiosis refer to changes in the mucous membrane directly affected and to remoter, more indirect disturbances. The local lesions caused by the invasion of epithelial

cells in the early stages are insignificant. The host cells retain their normal appearances, except as to size, and inflammatory reactions are absent. When the parasites have become widely diffused and occupy most of the cells, a number of changes are evident. Under a low power the tubules are no longer normal in form. Some appear missing. Under a high power the missing tubules are accounted for by the fact that most if not all of the epithelium has disappeared and the lumen is filled with cell elements chiefly polynuclears. Owing to the absence of a surface epithelium in the material available it is not clear that this epithelium has been invaded, but the presence of cell parasites in tubules up to the surface makes it probable that it also is attacked sooner or later. The loss of surface epithelium accounts for the small masses of leukocytes and fibrin and red blood corpuscles which cling to the denuded surface in places and represent an exudation and also the source of the hemorrhages (Fig. 7). These masses have embedded in them many oocysts. The hemorrhage is thus the aggregate of many small hemorrhages from the capillaries near the surface and this explains why the source of the blood is not macroscopically evident at autopsy.

A further lesion found in the advanced cases is a complete necrosis of a limited territory of the mucosa which involves up to ten or more tubules. In this focus cell nuclei have either completely disappeared or are broken up into fragments or pyknotic. The cytoplasm takes a distinctly reddish tint with eosin.

The more remote disturbances due to intestinal coccidiosis may be looked upon as due to infectious agents gaining entrance into the lymph and blood vessels of the denuded and only feebly reacting mucosa. Owing to the frequent association of pneumonia with the cases studied, injuries other than local are not definitely ascertainable. The pneumonia with one exception was associated with a microorganism to be discussed at another time as the probable etiological factor. In the exceptional case the pneumonic lesions were associated with streptococci and staphylococci. Of the five cases in which large numbers of coccidia were found either in the feces or in the epithelium of the large intestine or in both, probably three died as a result of the coccidiosis. Further studies upon calves kept under careful clinical observation will be needed to properly evaluate the disease as in itself a cause of death in this climate.

Brief Notes on Individual Cases.

Calf 34.—Red and white, female. Born Feb. 23, 1917.

Mar. 28. Coughing considerably. Reported to have had diarrhea several days ago. Mar. 30. Temperature 40°C. Apr. 6. Thin and languid. Cough and dyspnea. Feces liquid, of a greenish yellow color. Abdomen distended. Apr. 10. Temperature 40°C. Skin dry; hair roughened. Yellowish mucous discharge from nose. Discharges watery, with mucus tinged with blood. Apr. 12. Temperature 39.8°C. Growing thinner. Coughing readily induced. Fecal discharges are soft and contain clots of blood. Apr. 13. Blood passed in feces.

Apr. 14. Killed and autopsied. Weight 80 to 90 pounds. Besides consolidation of the cephalic half of lungs which will be discussed more fully at another time, there were no noteworthy lesions. The large intestine contained a rather offensive pea soup-like fluid and the flask-shaped glands below the ileocecal valve were distended with whitish mucus-like masses, but no lesions were detected to account for the blood passed in stools.

Histological examination of pieces of tissue from the small and large intestines fixed in Zenker's fluid and alcohol showed the following: At various levels of the small intestine a few coccidia are detected. They occur in groups. One to four may be seen in a tubule among many tubules not invaded. The distal portions of the villi appear to be free. The parasites are mostly in the sporont stage, as macrogametes, microgametocytes, and oocysts. The oocysts measure 20 to 22 microns long and 12 to 14 microns broad. A small number of tubules contain leukocytes both neutrophil and acidophil.

In sections from four different regions of the large intestine one focus was found within which the epithelial cells of the tubules were nearly all invaded by schizonts (Fig. 4). In intertubular tissue several empty oocyst shells were found.

Calf 36.—Red and white, female. Born Feb. 21, 1917.

Attention was first drawn to this case on Apr. 12 when it was noticed that large amounts of blood had been passed per rectum. Temperature 39.3°C. Apr. 13. Blood still present in feces and staining the anal region, tail, and legs. Temperature 39°C. Apr. 15. Calf reported very sick by attendant last evening. Found dead this morning.

The autopsy was not made until early, Apr. 16. The adipose tissue was abundant. The only recognizable lesions were localized congestion associated with punctiform hemorrhages in the lower small intestine, and similar but faded hemorrhages in the large intestine. Unfortunately, only one piece of the large and of the small intestine was saved for microscopic examination.

In the section of the small intestine every third or fourth tubule contained one to several oocysts; gametes were less numerous and schizonts scarce. The measurements indicate that elliptical coccidia are present. In the section of large intestine every tubule is invaded with coccidia chiefly in the stage of schizonts. A few oocysts are, however, present. Sections of the various viscera were negative.

Calf 49.—Red and white, female. Born Mar. 27, 1917.

Attendant called attention to this case stating that the animal had had diarrhea and blood in the discharges beginning Apr. 26, and that on Apr. 30 large amounts had been passed. On this date feces were watery and stained with blood. Tenesmus. Calf active and in good flesh. Salivation. Temperature 39.4°C. May 1. Temperature 38.4°C. Fecal blood still in evidence. Diarrhea diminished. Tenesmus increased. Slight salivation. Calf found dead in the morning of May 3.

The changes found at autopsy are briefly as follows: Mucosa of duodenum and upper jejunum sprinkled with punctiform hemorrhages aggregated into patches. Lower down there are uniformly and deeply congested patches. Moderate congestion of large intestine, except in lowest portion of rectum where it is more intense. The cephalic half of both lungs is sprinkled over with dark to bright red lobules interspersed among air-containing tissue. When the lobes are incised the central portions are found largely hepatized, the involved tissues showing dark red and lighter areas intermingled. Small whitish sclerotic foci permeating cortex of both kidneys.

Microscopic examination of fixed and hardened tissues shows foci of coccidia. These are minute and the number of parasites is not large. The prevailing forms are macrogametes, microgametocytes, and oocysts. These are of the smaller elliptical type. The infection varies greatly in intensity.

Sections from certain regions involving the entire circumference of the tube contain but a few parasites. In one section fifteen oocysts blocked one tubule. In other tubules nearby the lumen was full of debris including empty, collapsed oocysts and among them leukocytes. Only a few schizonts were detected.

In the large intestine (only one piece of tissue was available) every tubule contains fully formed masses of merozoites, which greatly predominate over the gametes in numbers.

The cause of death in this case was probably the septic pneumonia from which streptococci and staphylococci were isolated. The significance of the coccidiosis remains in doubt because only one small area of the large intestine had been preserved.

Calf 92.—Red and white, female; weight about 110 pounds. Born Apr. 24, 1917, found dead June 6.

The attendant reported that the calf had received some antidiarrhea serum soon after birth. Diarrhea, however, appeared for several weeks intermittently. June 4. Depression and loss of appetite were first noticed.

The autopsy, June 6, showed considerable postmortem changes. In the small intestine were localized congested areas. In the large intestine the mucosa had a dull, opaque appearance. Other organs appeared normal.

Microscopic examination of four different levels of the small intestine showed in each level scattering coccidia including schizonts, gametes, and oocysts. In three levels of the large intestine all epithelium still *in situ* was invaded. Thus in two levels there were chiefly the asexual stages with merozoites fully developed.

Oocysts were also present and indiscriminately scattered among the schizonts. In the third region oocysts predominated and some tubules were packed with the liberated forms. In this case the large ovoid oocyst was seen for the first time, and associated with the smaller elliptical form (Fig. 6).

Calf 102.—Holstein, female. Born Apr. 15, 1917.

May 1. Symptoms of pneumonia first appear. June 26. Emaciated; staring coat. Temperature 39.5°C. Cough, dyspnea, and slight nasal discharge. Fecal discharges normal. June 28. Killed. Calf weighs 100 to 120 pounds.

The autopsy shows extensive consolidation of lungs. Cephalic and ventral lobes symmetrically involved. Whitish, lardaceous foci in cortex of both kidneys. The digestive tract showed the following abnormalities: In the fourth stomach two erosions about 3 cm. in diameter covered with adherent thin, greenish-stained exudation. Some point-like hemorrhages in ileocecal valve and rectum. Oocysts of the smaller ellipsoidal type found in considerable numbers in feces pressed from rectum. In snippings from the fresh intestine none were detected.

In sections of the small intestine none were seen. In those of the large intestine a few gametes present in surface epithelium and several oocysts in phagocytes in intertubular tissue of mucosa. The cysts are partly collapsed. Evidently the process was nearly over.

Calf 121.—Red and white, female. Born June 25, 1917, found dead July 31.

The following details were obtained. The calf had lost most of its coat of hair at the time of death. The falling out of hair began early in July and was progressive in character. The animal had suffered from diarrhea intermittently but there was no evidence of blood-passing.

Body weighs about 70 pounds. Markedly emaciated. Skin almost free from hair; dry and harsh. Some ecchymoses and extravasations in subcutis. Gas blebs in axilla. Hair-balls embedded in large quantity of clear, viscid mucus in fourth stomach. Congestion of mucosa with scattering petechiæ. Throughout the small intestine small sharply defined hemorrhages more or less uniformly distributed. The large intestine showed nothing noteworthy. Consolidation of the ventral and the distal portion of cephalic lobe of the right lung with filling up of the air tubes of affected lobes with mucopurulent molds. Feces taken from rectum contained large numbers of oocysts of the ellipsoidal type.

Sections from six different levels of small intestine contained no coccidia. In the large intestine complete transections of the tube in three different regions revealed extensive invasion with coccidia. In one section every tubule was involved. The parasites were chiefly in the stage of asexual multiplication with a fair number of gametes and oocysts in the tubules. Many of the latter were packed with necrotic cells. In one tubule could be seen merozoites, oocysts, macro- and microgametocytes. In a second cross-section one large focus was found, the remainder of the tubules being filled with mucus-secreting cells. In the invaded area there were chiefly merozoites with a fair number of microgametocytes. The third section contained three foci of invasion in which each epithelial cell contained one to three schizonts. Gametes very rare. Decline

and death in this calf were probably due to the extensive invasion of the large intestine.

Calf 139.—Guernsey, female. Born Aug. 15, 1917.

Sept. 25. Cough first noticed. Sept. 28. Temperature 41°C. Feces soft, approaching diarrhea. Oct. 2. Removed from herd and killed because of physical signs of pneumonia.

At autopsy there was found symmetrical consolidation of the cephalic half of both lungs. The mucosa of small intestine was more or less congested throughout. Below the valve in the large intestine the patch of flask-shaped glands is deeply congested. Yellowish, soft, cheesy plugs fill the lumina of glands. Through remainder of colon scattering, point-like hemorrhages. In rectum congestion well marked. Fresh clippings of mucosa taken from valve to rectum show presence of large, ovoid oocysts in all preparations. In sections of fixed tissue, however, only a few oocysts found and these in intertubular tissue. Evidently the process had run its course.

Calf 184.—Red and white, female. Born Oct. 1, 1917.

Between Oct. 20 and 23 temperature fluctuated between 40° and 41°C. Oct. 23. Blood in feces, but coccidia not found among blood cells. Tenesmus. Dec. 5. Calf found dead. Probably *B. coli* septicemia. In feces from rectum and in snippings from mucosa of large intestine near valve a few large ovoid oocysts found. In sections from various levels of the small intestine a few gametes were detected.

Recovery from coccidiosis is illustrated by the following cases.

Calf A.—Born July 12, 1917. When 32 days old, on Aug. 13, blood appeared in the discharges associated with large numbers of oocysts. Aug. 22. Feces were again examined and one oocyst was found. Subsequent history uneventful. The calf was normal in size in Jan., 1918.

Calf B.—On Aug. 15, 1917, when the calf was 39 days old, blood appeared in feces. A considerable number of large ovoid and a few smaller elliptical oocysts were found. Jan. 17, 1918. This calf is in normal condition as regards weight.

Calf C.—Aug. 15, 1917. 32 days old. Several oocysts found. Jan. 31, 1918. Of normal size.

Calf D.—Born Aug. 11, 1917. Sept. 4. One oocyst was found in feces. This calf was not affected clinically. Jan. 31, 1918. Of normal weight.

Calf E.—Black and white, female. Born Sept. 5, 1917. Oct. 3. Diarrhea started. Oct. 5. Feces of a penetrating putrefactive odor, containing blood. Microscopic examination shows in the blood clot many ovoid as well as elliptical oocysts. Jan. 17, 1918. Calf in very good condition.

Calf F.—Yellow and white, female. Born Sept. 5, 1917. Oct. 8. Diarrhea and blood in stools noticed. Tenesmus. In the feces containing many red corpuscles are large numbers of elliptical oocysts. Jan. 17, 1918. Calf in very good condition.

In addition to the cases recorded there were seven other calves whose feces were subjected to examination with negative result. Some of these were suffering from diarrhea, others not. This fact is of interest since these calves had been in the same yard with cases of coccidiosis. It is, of course, not improbable that repeated examination might have shown coccidia in all cases.

SUMMARY.

Discharges of blood per rectum, associated with oocysts of coccidia, were observed occurring in young calves during the warmer season of the year. In a small percentage of the cases death was probably due directly to the coccidiosis. Although the disease, known as red dysentery in Switzerland, may have existed in this country for some time, there seems to have been no knowledge of its existence and no reports of it have thus far been published. The coccidia have been artificially cultivated and shown to produce four spores. Two oocysts of quite different dimensions and having minor differential characters were encountered in the same animal in several instances.

The invasion of the epithelium of the small intestine was slight. The chief seat of the parasitism was the large intestine. The lesions following the loss of epithelium were superficial hemorrhages and filling up of the denuded tubules with polymorphonuclear leukocytes.

Addendum.—During 1918, up to May 31, only one case of bloody dysentery was observed in the same herd. This occurred in a calf over 11 months old. The coccidia found in the feces were of the smaller elliptical type. The symptoms rapidly disappeared.

The clinical data in this communication were gathered by Dr. Ralph B. Little.

EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. Blood in feces from a calf which subsequently recovered. The oocysts as contained in the fresh blood clot. $\times 300$.

FIGS. 2 and 3. Oocysts of the smaller coccidium from a culture showing the presence of four sporoblasts. Photograph retouched.

PLATE 2.

FIG. 4. A tubule from the upper colon of Calf 34 fixed in Zenker's fluid. Almost every cell contains one to three parasites. They are indistinctly seen between cell nucleus and free margin of the epithelial cell. The accentuated small spheres within the parasites are nuclei of future merozoites (trophozoites). $\times 750$.

FIG. 5. A tubule from the same focus of the colon of Calf 34 as that shown in FIG. 4. In this tubule the schizonts have broken up into groups of merozoites. The outline of the tubule is no longer recognizable. $\times 750$.

PLATE 3.

FIG. 6. Large intestine of Calf 92. A large and a small oocyst in the same field. The smaller one is clearly located in a tubule, the large one in the intertubular tissue. It is not improbable that in this instance owing to the toughness of the shell it may have been dislocated in cutting or mounting the section. $\times 750$.

FIG. 7. Large intestine of Calf 121. Note a mass of exudate adhering to the mucous membrane. The mass, made up of fibrin, blood corpuscles, leukocytes, and mucus, contains the ellipsoidal oocysts. The tubular epithelium is largely destroyed and the tubules are filled with polynuclear cells. $\times 90$.



FIG. 1.

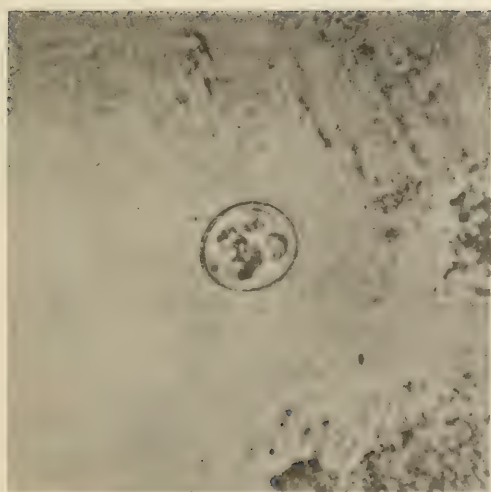
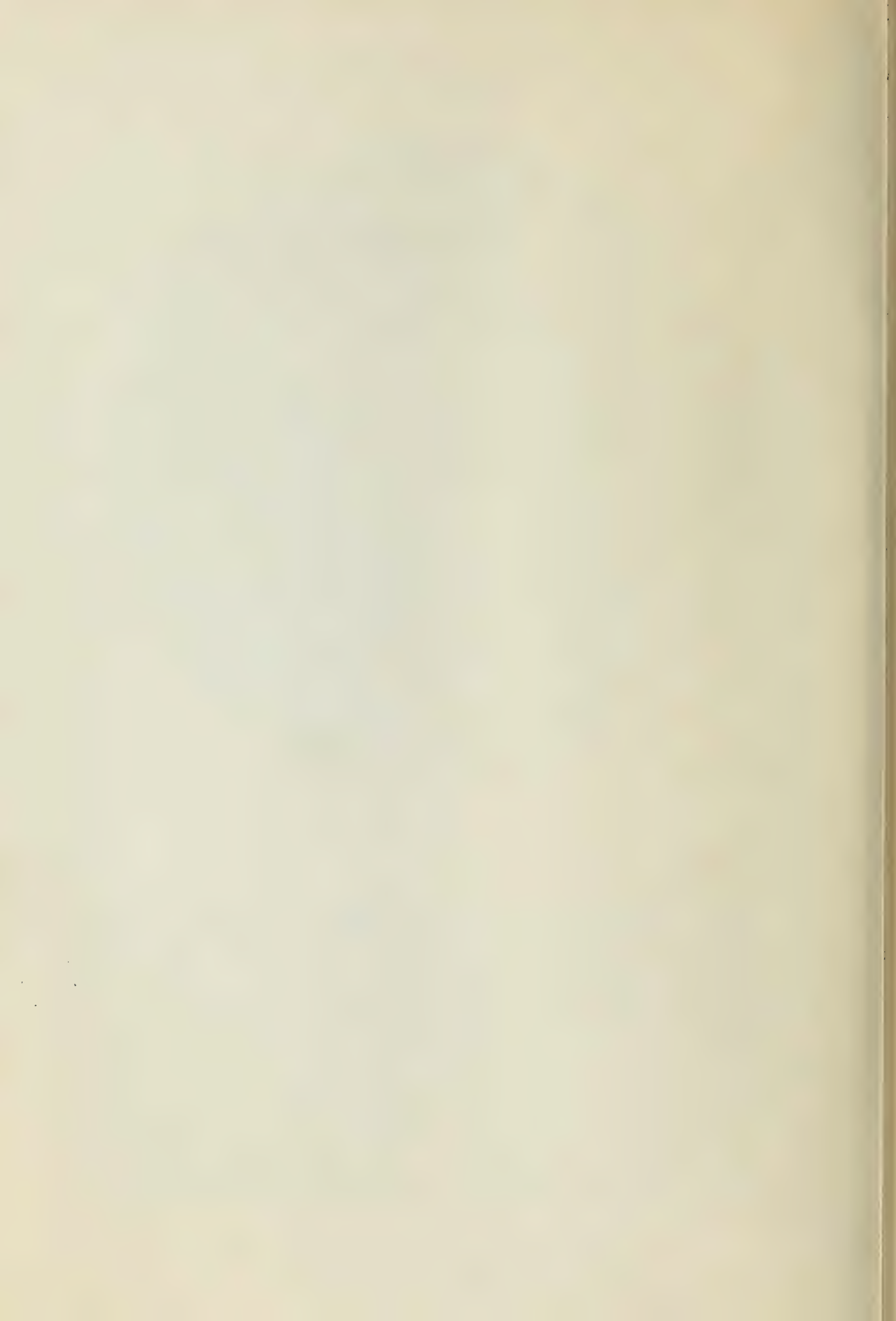


FIG. 2.



FIG. 3.

(Smith and Graybill: Coccidiosis in calves.)



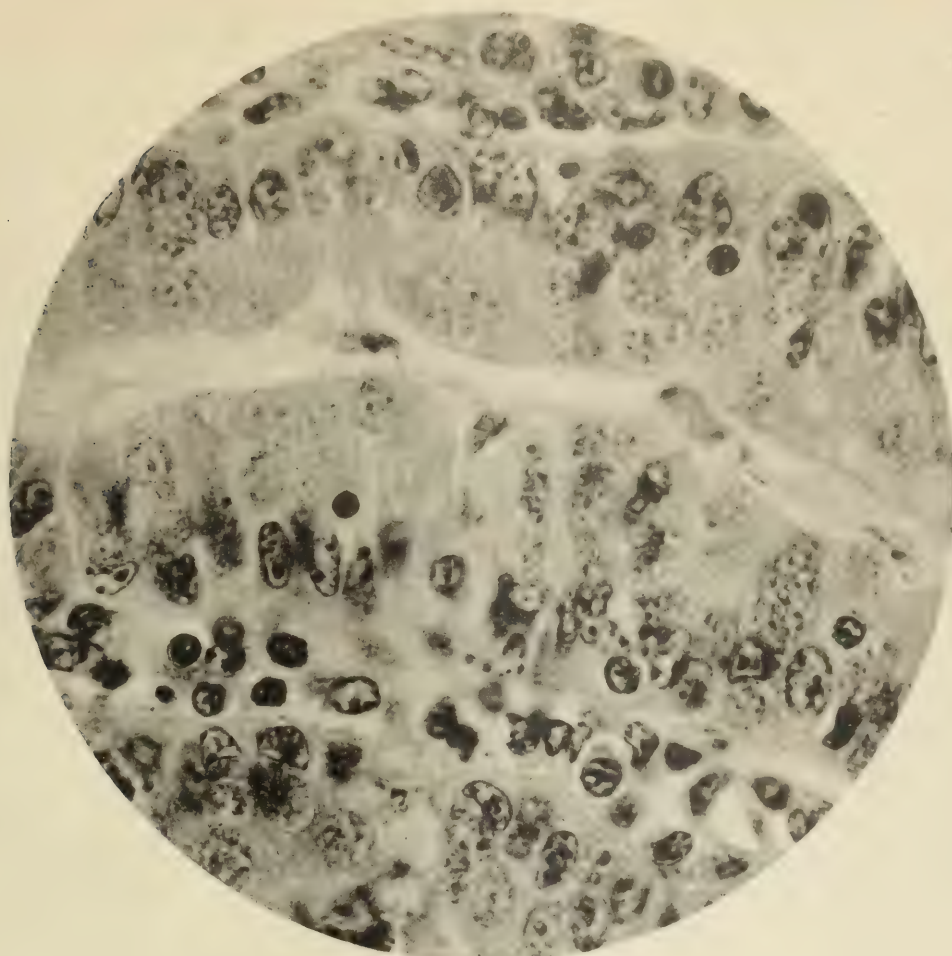
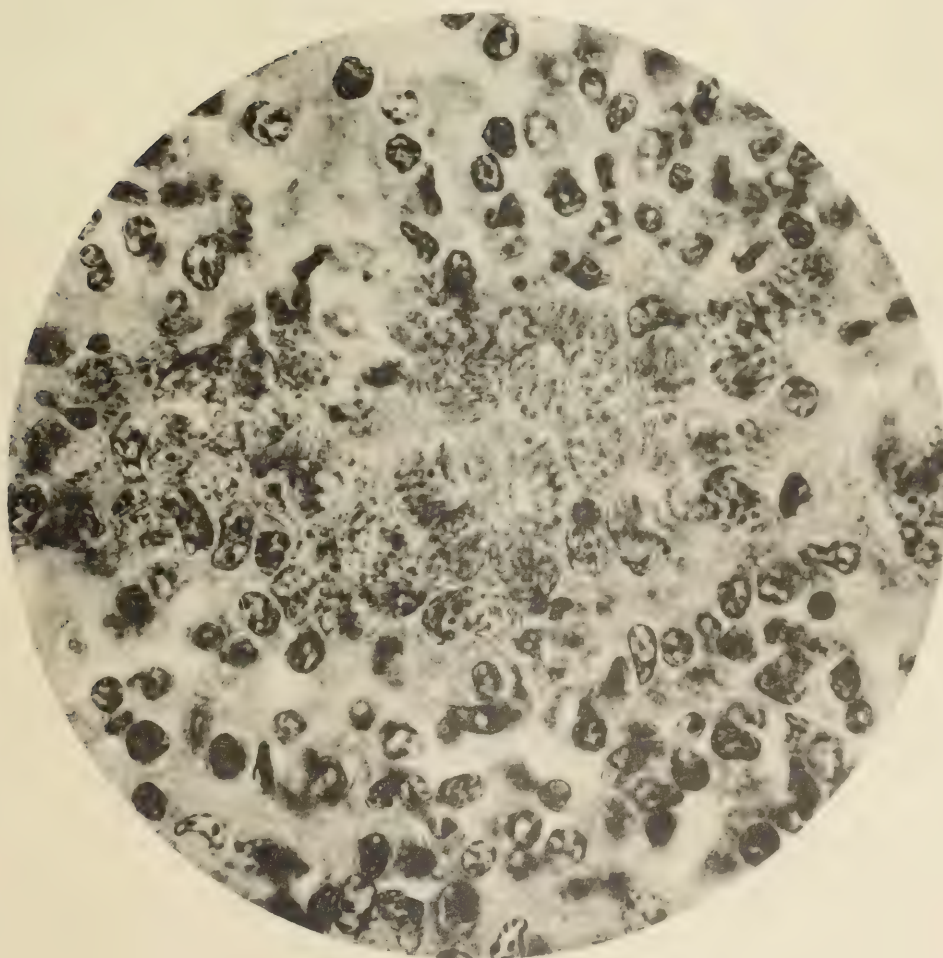


FIG. 4.



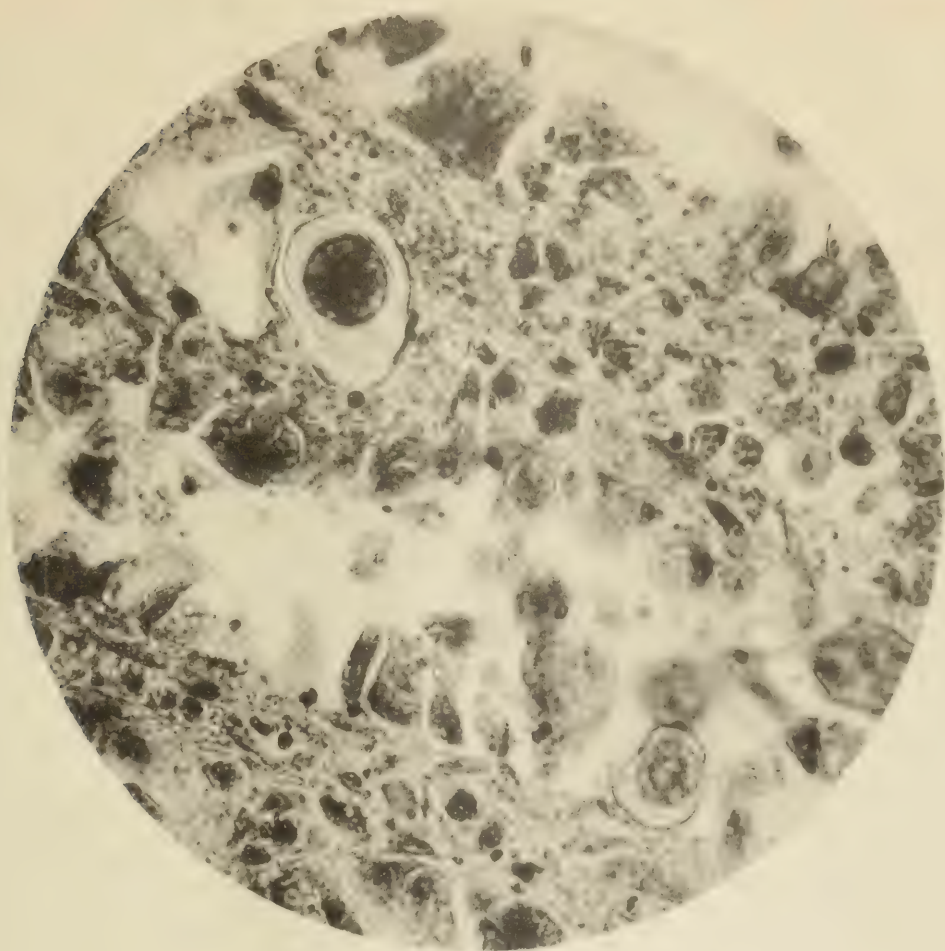
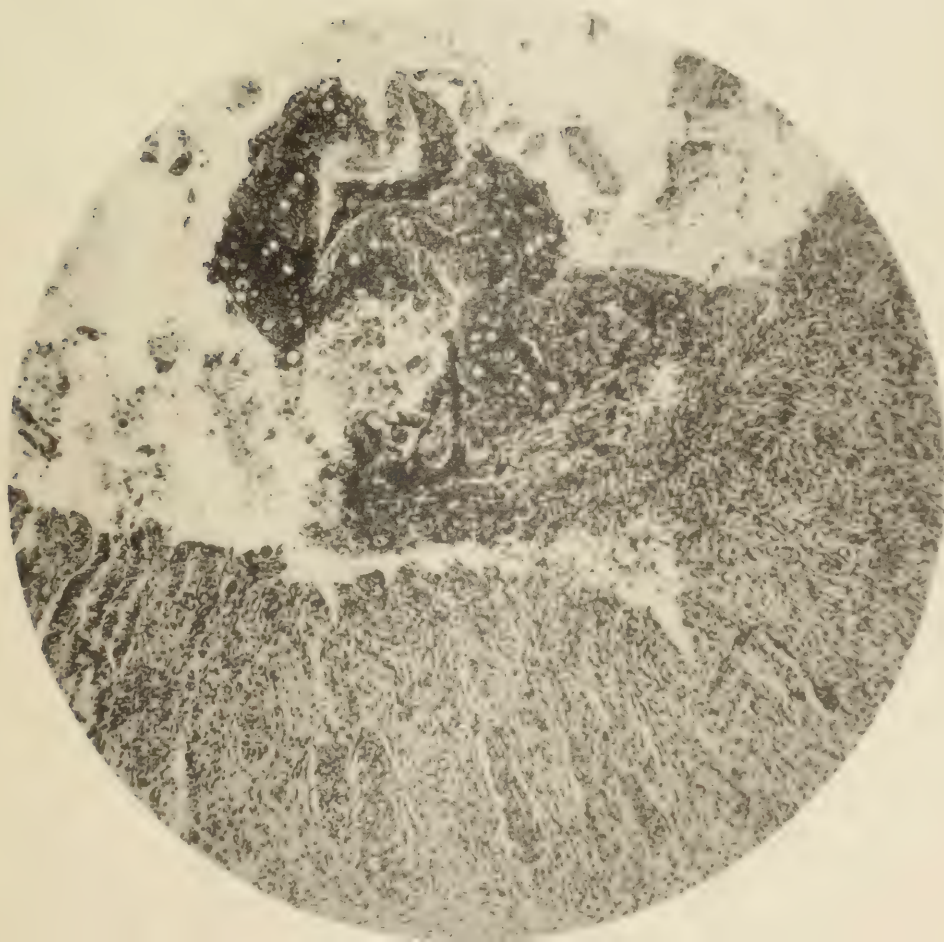


FIG. 6.



STUDIES IN BOVINE MASTITIS.

I. NON-HEMOLYTIC STREPTOCOCCI IN INFLAMMATION OF THE UDDER.

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INTRODUCTION.

Mastitis is one of the more important diseases affecting cows. Not only is it of serious nature economically, but within the past few years it has become of interest from the aspect of public health. Much of the recent work has tended to show that certain streptococci isolated from inflamed udders are closely related to if not identical with those found in certain epidemics of sore throat. Smith and Brown,¹ Davis and Capps,² and others believe, however, that these streptococci are of human origin and gain access to the udder through the teat canal.

Nocard and Mollereau³ appear to have been the first to undertake a study of the etiology of bovine mastitis. They succeeded in isolating streptococci from milk obtained from the udders of ten cows suffering from mammitis. The streptococci are described as growing in long chains in bouillon and staining well with Gram's method. In addition they state that the streptococci fermented sugar media.

Lucet⁴ examined milk from twenty-one animals affected with mastitis. From twelve he obtained non-gelatin-liquefying, Gram-negative bacilli; seven were infected with gelatin-liquefying, Gram-positive micrococci. The other two were suffering from streptococcic infection of the udder.

Guillebeau⁵ found ten species of organisms associated with infections of the mammary gland. He grouped them as follows: three species of non-gelatin-

¹ Smith, Theobald, and Brown, J. H., *J. Med. Research*, 1914-15, xxxi, 455.

² Davis, D. J., and Capps, J. A., *J. Infect. Dis.*, 1914, xv, 135.

³ Nocard and Mollereau, *Bull. et mêm. Soc. centr. méd. vét.*, 1884, 188.

⁴ Lucet, *Rec. méd. vét.*, 1889, vi, series 7, 423.

⁵ Guillebeau, *Landwirtsch. Jahrb. Schweiz*, 1890, iv.

liquefying micrococci, one group of gelatin-liquefying cocci, two species of streptococci, three kinds of bacilli producing gas in sugar gelatin, and a single strain of rods liquefying gelatin. The results of his bacteriological findings in 85 cases may be given as follows:

<i>Staphylococcus mastitidis</i>	33 times.
<i>Galactococcus versicolor</i>	12 “
“ <i>fulvus</i>	5 “
“ <i>albus</i>	2 “
<i>B. guillebeau</i> a.....	20 “
“ “ b.....	once.
“ “ c.....	“
<i>Streptococcus mastitidis sporadicæ</i>	8 times.
“ “ <i>contagiosæ</i>	3 “

Jensen⁶ subsequently identified *B. guillebeau* as *B. coli*. In addition he obtained *B. coli communis* and *B. lactis aerogenes* from several cases of garget.

Steiger⁷ undertook a detailed study of the disease and in all examined forty-five cases in cows and one in a goat. The summary of the bacteriological findings may be given as follows:

<i>Staphylococcus mastitidis</i>	6 cases.
Galactococci.....	10 “
Streptococci.....	10 “
<i>B. coli</i>	14 “
Mixed infections, <i>B. coli</i> , streptococci, and <i>B. necrophorous</i> ...	6 “

In addition, he had ample opportunities for clinical observation and was able to obtain considerable material for histological study. He discusses the possible modes of infection and considers that the usual mode is through the milk duct. It is admitted, however, that localization of a specific infection in the mammary gland may be caused by metastasis through the blood stream or lymph channels. The possibility of wound infection is also considered.

Unfortunately at the time of Steiger's investigation the differentiation of streptococci by their action upon carbohydrates and hemoglobin had not become a common practice. In describing both the streptococci and micrococci he employed grape and milk sugar. The streptococci were described as having a diameter of 1 micron. They grew in bouillon as diplococci or in short or long chains. Some grew diffusely throughout the medium, others left it clear. In agar they grew as small punctiform colonies. White mice were used to test the pathogenicity of the isolated streptococci. Some strains were highly virulent, others less so, and some produced apparently no ill effect.

⁶ Jensen, C. O., *Ergebn. allg. Path. u. path. Anat.*, 1897, iv, 830.

⁷ Steiger, P., *Centr. Bakteriolog., 1te Abt., Orig.*, 1904, xxxv, 326, 467, 574.

Prior to Savage's⁸ studies of microorganisms in mastitis, attempts to differentiate many of the organisms by more recent methods had not been made. Many believed that all streptococci isolated from inflamed mammae were identical. Savage appears to have been the first to undertake a study of the action of bovine streptococci upon carbohydrate media. He observed many strains isolated from the milk of normal cows and from the milk of cows suffering from inflammation of the udder. Inoculations of white mice were resorted to in order to establish pathogenicity. Mention is not made of the action of any strains upon hemoglobin. Examinations of milk from thirty-one cows afflicted with mastitis were recorded as follows:

Due to streptococci.....	21 cases.
“ “ staphylococci.....	5 “
“ “ <i>B. coli</i>	1 case.
“ “ <i>B. tuberculosis</i>	1 “
Of doubtful origin.....	3 cases.

Savage did not consider the problem from its economic standpoint but from the relation of the disease to that of the public health. The more important points in the clinical data of the cases were available and his cytological studies were of considerable value.

Henderson⁹ examined the secretions from fourteen cases of mastitis. Usually the examinations were made late in the course of the disease. Two were tuberculous, two were of the purulent type, and the remainder were described as parenchymatous. He believed that he was dealing with a mixed infection of streptococci and *B. coli* in twelve of the animals.

Zwick and Weichel¹⁰ succeeded in isolating *B. lactis aerogenes* from nineteen out of twenty-one cases of acute mammitis. From two individuals they obtained *B. paratyphosus*. Inoculation of the mammary glands of goats with these organisms produced severe inflammations of that organ.

Gilruth and Macdonald¹¹ also reported an outbreak of acute contagious mastitis caused by *B. lactis aerogenes*. They believe that this organism is not usually pathogenic to cattle when inoculated into the blood stream but it may gain entrance into the milk duct and set up serious inflammation of the mammary glands of lactating cows. Recently Ward¹² reports the isolation of *B. pyogenes* from udder lesions. In reviewing the literature on *B. pyogenes* he cites one reference with regard to its etiological relation to a certain type of mammitis.

⁸ Savage, W. G., *Rep. Med. Off. Local Gov. Bd. 1906-07*, xxxvi, 253; *Rep. Med. Off. Local Gov. Bd. 1907-08*, xxxvii, 359, 425; *Rep. Med. Off. Local Gov. Bd. 1908-09*, xxxviii, p. xxxiii.

⁹ Henderson, J., *J. Compt. Path. and Therap.*, 1904, xvii, 24.

¹⁰ Zwick and Weichel, *Arb. k. Gsndhtsamte.*, 1910, xxxiv, 391.

¹¹ Gilruth, J. A., and Macdonald, N., *Vet. J.*, 1911, lxvii, 217.

¹² Ward, A. R., *J. Bacteriol.*, 1917, ii, 619.

Kitt¹³ reviews the literature and classifies the disease etiologically according to whether it is caused by members of the colon group, of the paratyphoid and *enteritidis* groups, or of the groups of staphylococci and streptococci. He states that it is possible to observe many forms such as catarrhal, parenchymatous, and purulent inflammation, abscess formation, sclerosis, and a general rapid necrosis of the mammary tissue. It is pointed out that one type may succeed another, according to the period of lactation and the general resistance of the animal. Like Steiger and others he believes that the teat canal offers a ready method of access into the gland. Mention is also made of infections through the blood and lymph streams. In this connection he refers to Guillebeau and Hess¹⁴ experiment in which they injected *B. coli* subcutaneously into goats, which was followed by a localization in the mammary gland.

Etiological Studies.

The following studies were undertaken to define more accurately the species of organisms responsible for disorders of the mammary glands of cows, and if possible to lighten the economic burden imposed upon dairying by these affections. In addition, a more complete description of the biological characters of bovine streptococci obtained from inflamed udders seemed desirable for the purpose of assisting those interested in the public health problem of milk-borne epidemics of tonsillitis.

Much of the material has been obtained from a large dairy herd. Mastitis was more or less endemic. During 1916 it was necessary to dispose of 65 cows because of chronic mammitis. In addition to the actual loss from chronic cases, many animals developed milder forms of the disease generally evidenced by flocculi in the milk and inflammation of the gland. Milk from such quarters was discarded and represented an absolute loss. It is interesting to note that during 1916 the number of animals disposed of because of chronic mammitis doubled the number reacting to tuberculin. It has also been possible to obtain clinical data and samples of milk from other sources.

The following routine procedure is used in obtaining milk from inflamed udders. Milk from the affected quarter is drawn directly into a sterile 6 ounce wide mouthed bottle, and except in winter it is

¹³ Kitt, T., in Kolle, W., and von Wassermann, A., *Handbuch der pathogenen Mikroorganismen*, Jena, 2nd edition, 1913, vi, 96.

¹⁴ Guillebeau and Hess, cited from Kitt.¹³

iced at once. A note is made of the animal's general condition and the appearance of the involved quarter. The animal's herd number and its location in the various barns are recorded. The milk is usually plated within a few hours in dilutions of 1:10, 1:100, and 1:2,000. Culture medium employed in all initial examinations consists of 1 cc. of defibrinated horse blood and 12 cc. of 2 per cent veal bouillon agar, to which is added the diluted milk, and the whole plated. The effect on hemoglobin is noted at the end of 24 and 48 hours. Readings are always made from deep colonies.

In counting the leukocytes and other cells in milk a modification of the Doane-Buckley¹⁵ method has given satisfactory results. The original method may be described briefly as follows: 10 cc. of milk are poured into a graduated centrifuge tube and centrifuged rapidly for 10 minutes. The fat and supernatant liquid are drawn off down to the 1 cc. mark. A little saturated alcoholic solution of methylene blue is added and mixed. The counts are made with a Thoma-Zeiss blood counter. Campbell¹⁶ modified the technique by washing the sediment several times with distilled water. He considers the stain unnecessary. Campbell's technique proved very satisfactory, although 0.9 per cent salt solution was substituted as a washing fluid. Stained films from the sediment of the centrifuged milk were prepared for microscopic study.

The examination of milk obtained from 81 animals suffering from various forms of mastitis has revealed the following bacterial associations:

With non-hemolytic streptococci.....	31
“ hemolytic “	17
“ mixed hemolytic and non-hemolytic streptococci.....	2
“ micrococci.....	24
“ <i>B. coli</i>	2
“ pleomorphic Gram-positive rods.....	4
“ <i>B. lactis aerogenes</i>	1
	<hr/> 81

¹⁵ Doane, C. F., *Maryland Agric. Exp. Station, Bull. 102*, 1905.

¹⁶ Campbell, H. C., *U. S. Dept. Agric., Bureau of Animal Industry, Bull. 117*, 1909.

Early in the investigation it became apparent that non-hemolytic streptococci were responsible for many udder infections, and it was decided to study several infections of this type. Among these the following cases represent typical spontaneous infections.

Case Records.

Cow 55.—Holstein cow, age about 6 years. Said to have had an attack of mastitis in Jan., 1917. Date of parturition unknown. Developed mastitis in the left hind quarter on May 20, 1917.

May 21. First examination of the milk from the affected quarter. The quarter was enlarged, very firm, hot, and painful when manipulated. The walls of the milk duct were thickened. Milk could only be expressed with great difficulty in a very fine stream. A slight rise in temperature was recorded (102.4°F.). The milk was watery and contained many irregular flattened, white flocculi.

Agar plates prepared from milk of the affected quarter revealed 3,600 non-hemolytic streptococci per cubic centimeter. Attempts to ascertain the number of cells failed because they clumped and formed an unbreakable viscid mass after centrifugation. Examination of films from the milk sediment revealed streptococci in chains up to nine cocci and great masses of polymorphonuclear leukocytes. The other quarters appeared normal.

May 23. The milk was less watery but contained many fine flocculi in suspension. It contained 16,950,000 cells per cubic centimeter and the plates revealed 47,000 streptococci per cubic centimeter.

May 25. The quarter was still firm but not feverish or painful. Milk was watery and contained very little fat; the flocculi were numerous. The animal's temperature was normal (101.4°F.).

Left hind quarter:

Cells 16,800,000 per cubic centimeter.

Colonies 5,000 " " " pure culture of non-hemolytic streptococci.

Right hind quarter:

Cells 185,000 per cubic centimeter.

Colonies 50 " " " no streptococci. The milk appeared normal.

Left fore quarter:

Cells 90,000 per cubic centimeter.

Colonies 510 " " " no streptococci.

Right fore quarter:

Cells 460,000 per cubic centimeter.

Colonies 2,100 " " " 95 per cent of the colonies were similar to those observed in plates from left hind quarter.

Films from sediment contained polymorphonuclear leukocytes and a few streptococci. The quarter appeared normal. The milk failed to show flakes.

Examinations were made at usually 3 day intervals, but as they failed to show marked differences the results of many will be omitted. It is interesting to note, however, that on May 28 the milk from the left hind quarter revealed 24,800,000 cells and 723,000 streptococci per cubic centimeter. The quarter had a tendency to become less firm from day to day and on June 8 the corded portion was confined to the lower half of the quadrant. The cells had fallen to 14,200,000 and the streptococci to 250 per cubic centimeter.

June 5. The blood serum of this cow in a dilution of 1:500 completely agglutinated cultures of streptococci from the affected quarter. The highest agglutination recorded was on Aug. 1 when the streptococcic suspensions were partially clumped in a dilution of 1:2,000.

June 8.

Left hind quarter:

Cells 5,300,000 per cubic centimeter.

Colonies 364,000 " " " pure culture of streptococci.

The quarter was still firm. The milk was less watery but still contained flocculi.

Right hind quarter:

Cells 110,000 per cubic centimeter.

Colonies 120 " " " no streptococci.

Left fore quarter:

Cells 110,000 per cubic centimeter.

Colonies 180 " " " no streptococci.

Right fore quarter:

Cells 1,620,000 per cubic centimeter.

Colonies 14,600 " " " pure culture of non-hemolytic streptococci.

The quarter failed to show inflammatory changes and the milk appeared normal.

This animal was under constant observation until July 12. The original swelling of the quarter had contracted into an irregular nodule about the milk cystern. From June 8 until July 12 the elimination of streptococci from the diseased quarter had been very irregular, once falling as low as 8,000 per cubic centimeter. A maximum cell count of 91,000,000 was recorded, although at one time the cells fell as low as 8,800,000 per cubic centimeter. The right fore quarter continued to harbor streptococci.

July 12.

Left hind quarter:

Cells 53,000,000 per cubic centimeter.

Colonies 160,000 " " " pure culture of streptococci.

Right hind quarter:

Cells 210,000 per cubic centimeter.

Colonies 460 " " " no streptococci.

Left fore quarter:

Cells 120,000 per cubic centimeter.

Colonies 340 " " " no streptococci.

Right fore quarter:

Cells 280,000 per cubic centimeter.

Colonies 16,600 " " " pure culture of streptococci.

It was possible to examine the milk from time to time throughout the period of lactation. The left hind quarter ultimately became smaller than the others. The secretion was greatly diminished. The milk continued to contain flocculi and streptococci could always be recovered from the agar plates. The right fore quarter harbored streptococci throughout the lactation period but never revealed abnormalities. The cow gave birth to a calf in December. Up to Feb. 1, 1918, signs of streptococcic mastitis failed to appear. The subject has been averaging 20 quarts of milk per day. Plates from the left hind and right fore quarter have not revealed the presence of streptococci, since the animal calved.

Cow 56.—Holstein heifer, lactating for the first time. The animal calved Dec. 19, 1916. Mastitis developed in the right hind quarter May 21, 1917.

May 23. The animal was slightly depressed and a temperature of 102°F. was recorded. The cow had refused all food; the rumen was impacted. The right hind quarter was tense, feverish, and tender, but not noticeably enlarged. The milk from this quarter was much decreased in amount and was extremely thick. The other quarters appeared normal but the milk flow had decreased.

The exudate from the right hind quarter contained 27,300,000 cells and 5,600 streptococci per cubic centimeter. The cells in the milk from the other quarters were well within normal limits and plates prepared from the milk failed to reveal streptococci.

This animal was under observation for 56 days. The quarter softened somewhat but failed to regain its normal appearance; the milk became less purulent but always contained flocculi composed of casein, fibrin, and leukocytes. The highest cell count was recorded on June 11, when 182,000,000 were noted. The plates on this day revealed 1,300,000 streptococci per cubic centimeter.

The other quarters did not become involved, and streptococci were not observed in the plates.

The cow was slaughtered on July 18 and the udder was obtained for further study. The right hind quarter was firm and a trifle smaller than the others. On section the larger milk ducts were practically filled with yellowish flocculent milk. The parenchymatous tissue was pinkish yellow in color and when freshly cut appeared dry and granular; within a short time milk began to exude from the cut surfaces. There was an increase in interlobular connective tissue. The other quarters appeared normal. Pieces of the involved quarter were fixed in Zenker's fluid. Sections for study were stained with methylene blue and eosin. Examination of these sections revealed considerable degeneration and necrosis of portions of the secreting epithelium. In certain lobules it appeared granular; the

nuclei were shrunken and often forced to one side of the cells. The lumen of such an acinus was usually occluded with milk containing many fat cells and polymorphonuclear leukocytes. The interacinar vessels were engorged with leukocytes and red blood cells. In other lobules the secreting epithelium had been blotted out; the acini appeared indistinct. All that remained of the original structure was the supporting framework, a few necrotic epithelial cells, and dense masses of leukocytes.

The epithelium of many of the small lactiferous ducts stained indistinctly. Much of it was degenerated and infiltrated with leukocytes. Leukocytes and fibrin comprised the contents of the lumen. Many of the larger ducts had suffered severely. Much of their lining epithelium had become necrotic or was badly degenerated. The degenerated portions stained poorly, the nuclei were shrunken, and leukocytes had invaded the epithelial cells. The subepithelial connective tissue contained large numbers of round cells. Fibrin, necrotic leukocytes, milk, and microorganisms filled the lumen of the ducts.

Mammary glands of several other cows suffering from infection with non-hemolytic streptococci have been studied. In some the lesions are much more marked than in others. In Cow 66, slaughtered early in the course of the disease, some lobules consisted of purulent masses. In others the central acini were necrotic, while the peripheral had been invaded with dense masses of polymorphonuclear leukocytes. Widespread degenerative changes of the secreting epithelium had occurred. The interlobular connective tissue was congested and edematous.

In the left fore quarter of Cow 72 streptococci were found in the milk 13 days before gross changes in the quarter were observed. 75 days later the animal was slaughtered. On gross examination of this quarter much of the epithelial structures of the upper third of the gland had been replaced by connective tissue. The infiltration had extended downward into the center of the quarter in the form of fibrous strands constricting and blotting out many of the lobules.

Cow 141 had suffered from the same type of infection. The disease was chronic. The principal lesions were confined to the larger milk ducts.

Cow 70 affords an excellent example of a severe type of infection with non-hemolytic streptococci. Both hind quarters and the right fore quarter became so severely involved that the animal was killed. Lesions similar to those found in the sections of the gland of Cow 56 were observed.

The milk from affected quarters often varies in different individuals. Its character is influenced by the stage and severity of the infection. Usually at the onset it is more or less watery in appearance and contains many irregular white flakes of casein, fibrin, and cells. The reaction is slightly alkaline to litmus. It fails to coagulate when boiled. In chronic cases the exudate is usually yellow and less watery. The particles are larger, elongated, and have a tendency to coalesce on standing. The reaction is alkaline. Boiling usually produces a prompt coagulation.

It was possible in one instance to study an early infection. The animal had been under observation for 10 days before mastitis developed in the left fore quarter. The first count, on November 12, revealed 750,000 cells and 5,500 streptococci per cubic centimeter of milk. The quarter appeared normal and the milk was unchanged. 4 days later the streptococci had risen to 86,000 per cubic centimeter. Gross changes could not be detected in the quarter. Mastitis developed clinically on November 25. The count on November 27 revealed 19,000,000 cells and 1,640,000 streptococci per cubic centimeter.

Rühm¹⁷ had noted a similar condition previously. In making routine bacteriological examinations from individual cows he was able to detect streptococci in considerable numbers before clinical signs of inflammation appeared.

Streptococci identical with those responsible for the more severe inflammations may gain access to udders and inflict little or no gross changes in the gland or its secretion. Cow 69 was suffering from an infection of the left fore quarter with *Bacillus lactis aerogenes*. The other quarters were not involved. The right hind quarter became invaded with streptococci. The cell count rose to 610,000 per cubic centimeter at the end of a week and 10,800 non-hemolytic streptococci were noted in a cubic centimeter of milk. 1 week later the cells had risen to 1,000,000. The streptococci fell to 7,300. At the end of 10 days the cell count remained stationary but the streptococci had disappeared. It was only after pouring a considerable quantity of this milk through a sieve having 100 meshes to the inch that flocculi could be detected. Clinical mastitis did not develop in this quarter during the succeeding 10 weeks.

¹⁷ Rühm, G., *Woch. Thierheilk. u. Viehzucht*, 1908, lii, 125.

Morphological and Biological Characters of Non-Hemolytic Streptococci from Inflamed Udders.

In Table I the morphological and cultural characters of forty strains of non-hemolytic streptococci obtained from cases of mastitis are recorded. Fermented veal bouillon containing 1 per cent of the various carbohydrates and other substances was used to test the fermentative action of the strains. The initial reaction of the media varied between 0.6 and 0.8 per cent acid to phenolphthalein. Tubes 1.5 cm. in diameter, containing 13 cc. of media were used throughout. The column of liquid varied in height from 6.5 to 7 cm. Titrations were made after an incubation of 5 days at 38°C. The figures under each column denote the net production of acid.

The surface colonies on agar are usually round, delicate, slightly raised, almost transparent, and measure between 1 and 2 mm. in diameter. Occasionally one observes larger, flattened colonies. The deep colonies are tiny, ovoid, or biconvex in appearance. A few strains when grown in horse blood agar plates have produced a narrow, green-tinted zone about the deep colonies.

Morphologically the individuals appear as spherical or slightly elongated cocci. The chain formation varies over a considerable latitude. All stain by Gram's method.

Freshly isolated strains have not proved pathogenic for rabbits weighing between 1,500 and 2,000 gm. 1 cc. of a 24 hour bouillon culture injected intravenously failed to produce symptoms in most instances. Two animals revealed slight irregularities in temperature for a few days subsequent to inoculation but localizations failed to develop. Davis¹⁸ isolated non-hemolytic streptococci from three cases of mastitis. He was able to produce joint localizations in rabbits only after the intravenous injection of the growth from two or more blood agar slant cultures.

From Table I it will be noted that the mastitis streptococci fall into two groups. The larger, composed of thirty-four strains, produces acidity in dextrose, lactose, saccharose, maltose, and salicin. The five individuals of the other group agree as to their general characters except that they fail to act upon salicin. The acid produc-

¹⁸ Davis, D. J., *J. Infect. Dis.*, 1916, xix, 236.

TABLE I.
Morphological and Biological Characters of Non-Hemolytic Streptococci from Inflamed Udders.

Strain No.	Grouping.	Gram's stain.	Growth in bouillon.	Milk.	Production of acid in.							
					Dextrose.	Lactose.	Saccha-rose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.
					per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
C.51C.	L.C.*	+	Turbid.	Firmly coagulated.	4.8	4.0	3.7	4.0	0.0	0.0	0.1	3.9
C.51D.	"	+	"	"	4.4	4.0	3.8	3.2	0.1	0.0	0.0	4.2
C.55L.H.Q.	M.C.	+	"	"	4.2	3.7	3.6	3.9	0.2	0.1	0.0	3.3
C.55R.F.	L.C.	+	"	"	4.5	4.0	3.9	3.8	0.2	0.2	0.0	3.0
C.56R.H.	M.C.	+	"	"	4.6	3.7	3.9	4.1	0.0	0.0	0.0	3.5
C.65A.	L.C.	+	Clear.	"	5.0	3.5	3.5	4.6	0.2	0.0	0.0	2.8
C.67D.	M.C.	+	Turbid.	"	5.3	4.4	4.5	4.5	0.1	0.1	0.0	2.9
C.66	S.C.	+	"	"	3.6	3.8	3.9	3.9	0.1	0.0	0.2	3.5
C.70R.H.	L.C.	+	Clear.	"	3.8	4.0	3.7	3.1	0.0	0.2	0.1	2.6
C.70L.H.	"	+	"	Partially	4.1	4.1	3.7	3.7	0.0	0.0	0.0	3.5
C.70R.F.	"	+	"	Firmly	3.5	3.8	3.2	3.2	0.1	0.1	0.0	3.0
C.68R.H.	M.C.	+	Turbid.	"	3.9	3.5	3.2	3.4	0.1	0.0	0.1	2.8
C.72B.	S.C.	+	Clear.	"	4.2	3.5	3.1	3.8	0.0	0.1	0.1	3.1
C.141	"	+	"	Partially	4.5	4.2	3.4	2.9	0.2	0.1	0.0	4.0
M.L.	M.C.	+	"	"	2.2	2.7	2.5	2.1	0.0	0.0	0.1	2.0
M.S.	"	+	Turbid.	Firmly	5.3	3.5	4.5	2.9	0.1	0.0	0.0	3.6
M.U.	"	+	Clear.	Partially	3.4	3.6	3.5	3.5	0.0	0.1	0.0	3.0
M.X.	"	+	Turbid.	Firmly	3.8	3.1	3.2	4.0	0.0	0.0	0.0	2.3
M.2	"	+	Clear.	Partially	2.3	2.9	3.2	2.5	0.1	0.0	0.0	0.2
M.5	S.C.	+	"	"	2.2	2.3	2.4	2.0	0.2	0.2	0.2	0.2
M.8	"	+	"	"	2.7	3.0	2.6	2.3	0.1	0.1	0.1	0.1
M.10	"	+	"	"	6.5	4.2	3.7	4.7	0.1	0.0	0.0	0.5

M.11	L.C.	+	Turbid.	Firmly coagulated.	4.9	3.8	3.8	5.0	0.0	0.0	0.0	2.9
Abs.4	M.C.	+	"	"	2.3	2.6	2.3	2.0	1.5	1.1	1.5	1.2
M.21	"	+	"	"	4.2	3.4	3.0	3.3	0.1	0.0	0.1	0.0
M.22	S.C.	+	"	"	4.4	4.5	4.1	4.0	0.1	0.0	0.0	2.4
M.25	M.C.	+	"	"	3.3	2.9	2.4	3.0	0.1	0.1	0.1	1.5
M.34	"	+	Clear.	Coagulated on boiling.	3.0	3.3	3.3	3.5	0.0	0.0	0.0	0.0
M.35	S.C.	+	Turbid.	Firmly coagulated.	4.9	4.4	4.1	5.0	0.0	0.0	0.0	3.9
M.37	M.C.	+	"	Coagulated on boiling.	3.3	2.5	2.8	3.3	0.0	0.0	0.0	1.9
M.38	"	+	Clear.	Firmly coagulated.	4.5	3.8	3.7	4.1	0.1	0.0	0.1	3.1
M.40	S.C.	+	"	"	5.6	3.8	4.3	4.9	0.1	0.1	0.0	3.1
M.42	L.C.	+	"	"	3.9	3.7	3.3	3.1	0.0	0.0	0.1	2.6
M.45	M.C.	+	"	"	3.6	3.5	3.4	3.2	0.1	0.0	0.1	2.4
M.46	"	+	"	"	3.9	3.5	3.4	3.5	0.1	0.1	0.1	2.6
M.58	"	+	"	"	4.0	3.6	3.6	3.4	0.0	0.1	0.0	2.6
M.61	L.C.	+	"	"	4.1	3.8	3.7	3.4	0.1	0.1	0.0	2.9
M.62	M.C.	+	Turbid.	"	4.2	3.6	3.6	3.5	0.0	0.0	0.1	3.4
M.73	"	+	"	"	4.2	3.9	3.9	4.0	0.0	0.0	0.0	3.7
M.75	"	+	"	"	4.0	3.7	3.7	3.5	0.1	0.0	0.0	3.3

* The length of the chains in bouillon has been indicated as follows: S.C., chains of six or eight elements; M.C., threads of ten to twenty cocci; and L.C., chains of more than twenty.

tion of Strains M.5, M.8, and M.2 in dextrose, lactose, saccharose, and maltose bouillon is uniformly lower than that generally recorded. Strain Abs. 4 differs markedly from the others in that it ferments all the carbohydrates, although acid production is lower in raffinose, inulin, mannite, and salicin than in the others. This streptococcus was isolated from a subcutaneous abscess of the udder of a cow.

All streptococci grew well in bouillon, some left the medium clear, others produced a diffuse turbidity. A large majority firmly coagulated milk, others produced only a partial clotting after incubation for 5 days. In two instances the medium appeared unchanged when removed from the incubator, but coagulated promptly on boiling.

Since all strains failed to show major differences in their cultural characters, it seemed well to test their interagglutinability with a serum obtained from the injection of a single strain of streptococci. A non-lactating cow was chosen as an experimental animal. Before commencing the injections the serum of the animal was tested for agglutinins against three typical strains of streptococci. Agglutinations were not observed at dilutions of 1:10. Immunization with killed cultures of *Streptococcus* C.55 was begun on October 15, 1917. The doses were increased gradually, and when it seemed that a resistance had been established, living cultures were inoculated. On December 12 the serum completely agglutinated the streptococci at a dilution of 1:20,000.

The following method was employed in testing the agglutinating properties of each strain. The growth from 24 hour agar slant cultures was suspended in sterile 1 per cent solution of sodium chloride. Usually the suspensions were agitated with a platinum loop to break up the larger particles. All suspensions were diluted with the salt solution to a uniform density. To each cubic centimeter of this test fluid varying amounts of immune serum were added. Readings were made after incubation for 24 hours at 38°C. A tube containing only the suspension was incubated as a control.

Bovine streptococci usually produce homogeneous suspensions in a 1 per cent solution of sodium chloride and do not tend to precipitate spontaneously during the 24 hour incubation period.

All strains are agglutinated to a greater or less degree by the anti-serum produced by immunization with a single species. The non-

TABLE II.

Agglutination Titer of Non-Hemolytic Streptococci Tested with a Serum Produced by the Immunization of a Cow with a Single Strain.

Strain No.	Dilutions.						
	1: 100	1: 500	1: 1,000	1: 2,000	1: 5,000	1: 10,000	1: 20,000
C.55	++++*	++++	++++	++++	++++	++++	++++
C.55R.F.	++++	++++	++++	++	+	—	—
C.56R.H.	++++	++++	++++	++++	+	—	—
C.51C.	++++	++++	++++	++++	++	+	—
C.65A.	++++	+	+	—	—	—	—
C.67D.	++++	++++	++++	++++	++++	+	?
C.66	++++	++++	++++	++++	++++	++	+
C.70R.H.	++++	++++	++++	++	+	—	—
C.70L.H.	++++	++++	++++	++	++	—	—
C.70R.F.	++++	++++	++++	++++	+	?	—
C.72B.	++++	++++	++++	++++	++++	++	+
C.68R.H.	++++	++++	++++	++++	++	—	—
C.51D.	++++	++++	++	+	—	—	—
M.L.	++++	++++	++++	++++	++	—	—
M.S.	++++	++++	++++	++++	++++	+	—
M.U.	++++	++++	++++	++	+	+	—
M.2	++++	++++	++++	++++	++++	++	+
M.5	++++	++++	++++	++++	++	+	—
M.8	++++	++++	++++	++++	++++	++++	++++
M.10	++++	++++	++	+	—	—	—
M.11	++++	++++	++++	++++	++++	++++	+
Abs.4	++++	++++	++++	++	—	—	—
M.21	++++	++++	++++	++++	+	—	—
M.22	++++	++++	++++	++++	++	+	—
C.141	++++	+	—	—	—	—	—
M.25	++++	++++	++	—	—	—	—
M.34	++++	++++	++++	++++	++++	++	—
M.35	++++	++++	++++	++++	++++	++	+
M.37	++++	++++	++++	+	—	—	—
M.38	++++	++++	++++	++++	++++	++++	++++
M.40	++++	++++	++++	++++	++++	++++	++++
M.45	++++	++++	++++	++++	++++	++++	++
M.46	++++	++++	++	+	—	—	—
M.58	++++	++++	++++	++	+	?	—
M.61	++++	++	+	—	—	—	—
M.62	++++	++	+	—	—	—	—
M.73	++++	++++	++++	++++	+	—	—
M.75	++++	++++	+	+	—	—	—

* Clumping attended by complete clearing of the fluid has been recorded as + + +. ++ indicates considerable agglutination without the entire clearing of the fluid. A moderate precipitation has been considered +. A negative reaction has been recorded as —.

salicin-fermenting strains—M.2, M.5, M.8, M.21, and M.34—were agglutinated as readily as those which attacked this substance (Table II). Streptococcus Abs. 4 which differed from all the others was likewise agglutinated at a serum dilution of 1:2,000.

All the streptococci except Strains C.141, M.58, M.61, M.62, M.73, and M.75 were isolated from cases of mastitis occurring on one farm. In each case agglutination occurred at a minimum serum dilution of 1:1,000. Of the six strains from other farms five agglutinated at 1:1,000, the other (Strain C.141) was partially clumped at 1:500. Cultures obtained from the same source as the immunizing strain were uniformly agglutinated at higher dilutions than those obtained elsewhere.

Five strains of hemolytic bovine streptococci isolated from inflamed udders were also tested. Each strain possessed many characters in common with the non-hemolytic types. Usually the only distinguishing difference was their action upon hemoglobin. In no instance were any of them agglutinated at dilutions as low as 1:100.

Feeding of Mastitis Milk to a Pig.

Although freshly isolated strains of non-hemolytic streptococci failed to produce marked effects when inoculated into rabbits, it was considered necessary to test the effect of ingestion of large quantities of these organisms. A young pig weighing 99 pounds was chosen for the experiment. It was fed for 15 days with flocculent milk from Cows 55 and 56. The animal averaged about 2 quarts of purulent milk a day. The diet was augmented with a small amount of grain. Morning and evening temperatures were taken before the feeding was begun and during the experiment. The slight variations recorded were well within normal limits. The animal was under observation for 10 days after the milk feeding was discontinued but failed to show symptoms of any disorder. The pig gained 25 pounds during the experiment.

DISCUSSION.

Mastitis caused by infections with non-hemolytic streptococci is more prevalent than that caused by other classes of microorganisms. Data point to the extreme severity of these infections. Of the thir-

teen animals under observation for a considerable period but four have recovered. The others have either lost the function of the involved quarters or the disease has progressed to such an extent that they no longer remained profitable as milk producers.

It has been the custom of many investigators to consider the entrance of pathogenic microorganisms into the mammary gland in three ways: (1) metastasis from another disease focus within the body; (2) through wounds; and (3) through the teat canal. Localizations through the blood and lymph vessels occur in tuberculosis and actinomycosis as well as in some other maladies. Wound infection is probably responsible for gangrenous forms of mammitis. The probable mode of infections caused by non-hemolytic streptococci is through the teat canal. The disease is local, usually only one or two quarters are involved, and the general condition of the animal is not markedly affected. The elimination of streptococci several days before symptoms develop also points to entrance through the duct of the teat.

Injury has been considered by many investigators to be an important predisposing factor in udder inflammation. This has not been my experience. If injuries occurred they were of such a minor nature that they escaped detection. If injury plays a major part as a predisposing factor one would expect to find, in many cases, ulceration of the lining membranes of the large ducts and milk cystern and abscesses of the parenchyma. Such lesions have not been observed in the material examined.

It has been difficult to trace infection from one animal to another. On the farm where a large proportion of the material was obtained a "gang" system of milking has been adopted. The milkers are each assigned a cow to milk. The attendant washes his hands after milking each animal, and he is assigned another. In this way each man milks two or three cows irregularly spaced about each barn. This procedure renders the tracing of infections difficult. It seems reasonable to suppose that the extreme irregularity of the occurrence of infection throughout the herd may be explained by the transfer of the virus on the hands of the milkers. Clinical cases are constantly appearing. Cows revealing gross changes in one quarter and harboring streptococci in apparently normal quarters must be considered as dan-

gerous virus reservoirs. Incipient cases eliminate streptococci before symptoms develop. One animal suffered from an udder invasion with streptococci identical in every respect with those obtained from severe inflammations but never developed clinical mastitis. These conditions account at least for the spread of the virus. Contamination of the ends of the teats with feces and vaginal secretions may explain other possible sources of infection.

On one farm visited the incidence of udder inflammations approximated 10 per cent of the cows in one barn. It was customary to milk with a milking machine. The general sanitary conditions were excellent, except that the teat cups were not disinfected or sterilized between the milking of individual cows. The infection was probably spread by the contaminated milk cups.

Definite evidence is lacking to show whether the non-hemolytic streptococci isolated from inflamed mammae are pathogenic for consumers of milk. Milk-borne epidemics of tonsillitis have been attributed to hemolytic streptococci and up to this time the non-hemolytic forms have not been incriminated, although it must be assumed that non-hemolytic streptococci from inflamed udders gain frequent access to the milk supply. The lack of virulence of these organisms when injected into rabbits and when milk containing enormous numbers of these streptococci was fed to a pig indicates their low pathogenicity for species other than bovines.

SUMMARY.

It seems clearly established that non-hemolytic streptococci are responsible for a considerable number of cases of bovine mastitis. Of the 81 animals examined, 31 were suffering from infections of this type. The lesions produced in invaded quarters varied from an involvement of only the lining epithelium of the large milk ducts to severe degeneration and necrosis of the secreting epithelium. In one instance a considerable portion of the glandular elements had been replaced with connective tissue.

The streptococci fall into two groups when their action on the various carbohydrates is considered. Thirty-four strains fermented dextrose, lactose, saccharose, maltose, and salicin; five others attacked

the first four sugars but failed to produce acid in salicin. All mastitis streptococci failed to act upon raffinose, inulin, or mannite. One species isolated from a mammary abscess produced acid in all the carbohydrates.

All the strains were agglutinated with an antiserum prepared from one typical strain. The agglutination titer varied over wide limits, although all the streptococci were agglutinated at a dilution of 1: 500. None of the strains inoculated proved pathogenic for rabbits. A pig fed on the milk from two typical cases of mastitis remained well.

Acknowledgment is due to Dr. Ralph B. Little, of this Department, who collected many samples of milk and certain clinical data during the investigation.

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